A STUDY OF THE EFFECT OF VITAMIN B AND IODINE ON THE WEIGHT, IODINE CONTENT AND STRUCTURE OF THE THYROID GLAND OF THE RAT

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FOUR FIGURES

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Cowgill ('32) and Hendricks ('33) have suggested that the vitamin B(B_1) requirement of an animal is proportional to its metabolism. The thyroid gland, through its iodine containing hormone (thyroxin), has been shown to provide a means both for maintaining a higher rate of metabolism than would otherwise be obtained and for varying the rate of metabolism to meet changing physiologic needs.

It has been shown that the function of the thyroid can be limited by a deficiency of iodine. McClendon ('27) and Orr ('31) have reviewed the subject of goiter and its relation to iodine supply. Studies of the effect on the thyroid of cabbage feeding (Webster and Chesney, '30), of methyl cyanide (Marine, Rosen and Cipra, '33), of calcium (Hellwig, '34) and of chlorides (Hibbard, '33) have indicated that a number of factors may increase the needs of the body for iodine or for the iodine containing hormone and thereby may play a part in the development of simple goiter.

A number of workers have associated thyroid function with the vitamin B complex and lately with the B(B_1) fraction. McCarrison ('14) noticed hyperplasia of the thyroid of pigeons fed a diet of polished rice. The suggestion has been made (Aalsmeer, '32) that beri-beri is associated with dysfunction of the thyroid. Stepp and György ('27) state that

vitamin B deficiency causes loss of colloid and hyperplasia of the cells lining the follicles. Experiments on both pigeons and dogs indicate that there is a relation between vitamin B requirement and metabolism (Cowgill and Palmieri, '33). A similar reaction has been pointed out by Hendricks ('33). McCance ('33) has stated that vitamin B along with iodine, is one of the essentials in the cure of certain goiters. According to Sandberg and Holly ('33) vitamin B causes the involution of goiter produced in rabbits by cabbage feeding, but not to the degree caused by iodine. Fischer ('33) concluded from microscopic findings that the antineuritic vitamin of unpolished rice produced the same histological effect on the thyroid as iodized salt.

Thus it is seen that a relation between vitamin B and the thyroid has been indicated but has not been studied critically. It was our purpose to make such a study, namely: the effect of vitamin B(B_1) on the thyroid of the rat as shown by weight, iodine content, and structure of the gland when the rat was fed well-controlled purified diets at different levels of iodine intake.

EXPERIMENTAL

The rats used in this study were bred on an adequate stock ration of the following composition: whole wheat flour, 30.0 parts; yellow corn meal, 30.0 parts; whole milk powder, 30.0 parts; linseed meal, 5.0 parts; alfalfa meal, 2.7 parts; wheat germ 1.0 part; calcium carbonate, 0.6 part; sodium chloride, 0.6 part. (A short time previously, iodized salt and 3 parts cod liver oil had been used, but these were discontinued because the young were getting such a large store of iodine.) Twenty-five animals were placed on each diet. Rats of both sexes, 26 days old and weighing between 60 and 70 gm., were used. Animals which were fed concentrates were kept in individual cages; otherwise two or three were caged together. Distilled water was given to all animals and a record of food consumption was kept for each cage.

With certain modifications iodine was determined by the Leitch and Henderson ('26) method. The food materials were ashed by the von Kolnitz and Remington ('33) method while

the thyroids were burned in a combustion tube 1 inch in diameter. It was found that good checks could be obtained with the combustion tube with sugar and potassium iodide and with samples of desiccated thyroid if the oxygen was passed through slowly. A tube of small diameter or a rapid flow of oxygen caused the smoke to be swept through, and check results could not be obtained.

Iodine determinations were made on the diet materials and on one lobe of the thyroid of each rat. Differences as small as 0.10 γ of iodine could be detected and in samples containing 0.5 γ or more the error was less than 10%.

At the end of the experimental feeding period the rats were killed with chloroform and the thyroid glands were weighed in a moist chamber. The glands were obtained by removing the trachea and thyroid intact and then pulling the trachea and attached muscles free, while the gland was held between the operating board and a scalpel which was forced between it and the trachea. If proper care is exercised the gland is obtained complete and free from muscle. One lobe was placed in formalin for sectioning, the other was dried and saved for iodine determination. The iodine content of the whole gland was calculated from the content of this one lobe.

The weight of the thyroid was recorded in three ways: 1) actual weight; 2) weight per 100 gm. of body weight; 3) per cent of Donaldson's ('24) normal values.

Three different age groups were studied as follows: 1) 41 days on experimental diet; 2) 82 days on experimental diet; 3) 123 days on experimental diet. Forty-one days were chosen as the initial period because that was the time necessary for our rats to show marked vitamin B deficiency. The other two periods were chosen as multiples of the initial 41-day period. The animals receiving the deficient diets were given some yeast supplement in order to sustain life for the longer periods.

Diets. The composition of the experimental diets and the groups into which they were divided are shown in table 1.

Results. The results are summarized in table 2, a study of which reveals some interesting findings that can best be brought out when discussed under the following headings:

TABLE 1

Showing composition of the diets and the groups into which they were divided

DIET NO.	GROUP NO.	VITAMIN FREE CASEIN[1]	AUTOCLAVED BREWER'S YEAST	BREWER'S YEAST (NOT AUTOCLAVED)	SUCROSE[2]	BUTTER-FAT	SALTS NO. 185[3]	VITAMIN G (B_2) CONCENTRATE[4]	VITAMIN B (B_1) CONCENTRATE[5]	KI	NATURE OF DIET
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*	
32	...	12.0	0	0	79.0	5.0	4.0	0	0	0	Def. in B complex and I_2
33	...	12.0	0	0	79.0	5.0	4.0	0	0	0.005	Def. in B complex 0.0038% I_2
38	I	12.0	5.0	0	74.0	5.0	4.0	0	0	0	Def. in B_1 and I_2
32G	I	12.0	0	0	79.0	5.0	4.0	+	0	0	Def. in B_1 and I_2
39	II	12.0	5.0	0	74.0	5.0	4.0	0	0	0.005	Def. in B_1, 0.0038% I_2
33G	II	12.0	0	0	79.0	5.0	4.0	+	0	0.005	Def. in B_1, 0.0038% I_2
39A	III	12.0	5.0	0	74.0	5.0	4.0	0	0	0.025	Def. in B_1, 0.019 I_2
40	IV	12.0	0	5.0	74.0	5.0	4.0	0	0	0.005	Complete diet, 0.0038% I_2
39B	IV	12.0	5.0	0	74.0	5.0	4.0	0	+	0.005	Complete diet, 0.0038% I_2
40A	V	12.0	0	5.0	74.0	5.0	4.0	0	0	0.025	Complete diet, 0.019% I_2
41	VI	12.0	0	5.0	74.0	5.0	4.0	0	0	0	Complete diet, no. I added
38B	VI	12.0	5.0	0	74.0	5.0	4.0	0	+	0	Complete diet, no. I added
32BG	VI	12.0	0	0	79.0	5.0	4.0	+	+	0	Complete diet, no. I added
33BG	IV	12.0	0	0	79.0	5.0	4.0	+	+	0.005	Complete diet, 0.0038% I_2

To all of the above diets 15 drops (2800 U.S.P. units) of viosterol were added per kilogram of food mixture.

[1] Casein purchased from Casein Manufacturing Company.

[2] Cane sugar used in preference to other carbohydrates because of its more uniform action in causing symptoms of vitamin B deficiency.

[3] McCollum's.

[4] Vitamin G(B_2) concentrate prepared from yeast according to the directions of Dr. N. B. Guerrant, as follows: One kilogram of yeast was extracted with water and alcohol added to the extract to make 50% by volume. The formed precipitate was discarded. Additional alcohol was added until a concentration of 80% by volume was obtained. The precipitate formed between these two concentrations was removed, dried, autoclaved to destroy any vitamin B present, dried again, and kept in a desiccator. An amount equivalent to 1 gm. of the original yeast was fed to each animal daily.

[5] The vitamin B concentrate was prepared from wheat germ by extracting with acidified (0.1% glacial acetic acid) 95% alcohol and evaporating the extract under reduced pressure. It was fed daily at a level equivalent to 1 gm. of the original wheat germ per rat. All fatty and waxy materials soluble in ether were removed from the wheat germ before leaching with alcohol.

All fatty and waxy materials soluble in ether were removed from the wheat germ before feeding with alcohol.

TABLE 2

Showing average iodine intake, body weight, thyroid weight, iodine content and concentration of thyroid

DIET NO.	GROUP NO.	IODINE CONTENT OF DIET	AVERAGE TOTAL INTAKE PER ANIMAL	INITIAL AND FINAL BODY WEIGHT	THYROID WEIGHT			IODINE CONTENT OF THYROID			
					Actual	Per 100 gm. body weight	Per cent of Donaldson's values	Actual average	Fresh weight basis	Dry weight basis	Per 100 gm. body weight
41 days on experimental diet											
		γ/kilo.	*γ*	*gm.*	*mg.*	*mg.*		*γ*	*per cent*	*per cent*	*γ*
38	I	<20	<2.1	70– 56	5.9	11.1	52	4.4	0.074	0.24	7.8
32G	I	<20	<3.8	59– 46	4.0	8.7	38	5.8	0.16	0.34	12.6
39	II	38220	6268	74– 57	4.9	8.6	40	4.8	0.099	0.29	8.4
33G	II	38220	6738	58– 45	4.0	8.9	38	5.68	0.14	0.35	12.6
39A	III	191000	43853	63– 66	5.9	8.9	44	10.13	0.17	0.53	15.3
40	IV	38220	8775	63–102	4.5	4.4	23	24.9	0.55	1.51	24.4
39B	IV	38220	12849	62–104	7.3	6.8	42	8.97	0.13	0.36	8.62
40A	V	191000	59515	61– 95	7.4	7.6	41	26.05	0.36	0.86	27.4
41	VI	<20	<6	70–138	5.2	3.8	21	6.64	0.13	0.36	4.8
38B	VI	<20	<6.5	63–107	6.8	6.5	37	8.98	0.14	0.35	8.4
82 days on experimental diet											
38	I	<20	<5.7	68– 68	6.4	9.9	49	4.6	0.069	0.17	6.8
39	II	38220	11580	75– 80	6.0	7.5	38	7.07	0.117	0.36	8.8
39A	III	191000	73535	63– 76	5.8	7.6	38	10.14	0.17	0.47	13.3
40	IV	38220	29773	75–163	7.1	4.4	26	23.27	0.33	0.78	14.3
39B	IV	38220	25683	62–123	7.4	6.0	33	8.20	0.11	0.30	6.7
40A	V	191000	137711	59–144	7.5	5.2	30	22.11	0.29	0.68	15.3
41	VI	<20	<12	66–165	8.8	5.3	32	8.82	0.10	0.24	5.3
38B	VI	<20	<13	60–118	7.5	6.3	35	9.20	0.12	0.34	7.8
Stock				266	13.8	6.1	34	7.50	0.053	0.17	2.8
123 days on experimental diet											
38	I	<20	<8	69– 90	6.9	7.6	39	5.70	0.083	0.22	6.3
39	II	38220	20218	72– 94	5.7	6.0	31	10.54	0.19	0.50	11.2
39A	III	191000	70479	61– 53	5.1	9.6	44	9.74	0.19	0.66	18.4
40	IV	38220	48883	68–193	8.6	4.6	27	32.6	0.38	0.83	16.9
40A	V	191000	213786	64–167	9.8	5.5	32	31.12	0.34	0.74	18.6
41	VI	<20	<20	67–169	9.1	5.8	35	17.7	0.18	0.57	10.5
Stock				319	18.0	5.8	38	9.3	0.053	0.19	2.9

a. Appearance of the thyroid gland. Rats in group I consistently showed glands that were dark red in color with engorgement of the associated blood vessels. The rats in all the other groups had glands that were either light red or very pale in color. The variations in appearance could not be correlated with any histological difference or with any difference in iodine content.

TABLE 3

Showing average thyroid weight and iodine content of rats on paired feeding experiments

DIET NO.	GROUP NO.	IODINE CONTENT OF DIET	AVERAGE TOTAL INTAKE PER ANIMAL	INITIAL AND FINAL BODY WEIGHT	THYROID WEIGHT			IODINE CONTENT			
					Actual	Per 100 gm. body weight	Per cent of Donaldson's values	Actual average	Fresh weight basis	Dry weight basis	Per 100 gm. body weight
Average of three sets of litter mates											
		γ/kilo	γ	gm.	mg.	mg.	%	γ	%	%	γ
38	I	<20	2.5	67–55	3.9	7.8	35.1	4.7	0.12	0.28	8.5
39	II	38220	4777	63–45	3.7	8.6	35.9	4.3	0.12	0.34	9.6
40	IV	38220	4777	64–45	4.4	8.5	42.7	4.7	0.11	0.26	10.4
41	VI	<20	2.5	64–45	3.8	8.6	37.5	4.4	0.12	0.27	10.0
Average of three sets of litter mates											
32G	I	<20	1.7	64–39	4.9	12.4	53.4	3.9	0.09	0.30	10.0
33G	II	38220	3248	62–39	4.6	11.6	50.1	4.1	0.09	0.30	10.5
33BG	IV	38220	3248	60–42	4.7	9.9	42.9	4.5	0.10	0.31	10.7
32BG	VI	<20	1.7	61–50	4.1	9.8	42.5	5.0	0.11	0.34	10.0

b. Comparison of fresh thyroid weights. Forty-one-day period. When the groups are compared on the basis of the average actual weight of the gland it is found that there is not a statistically significant difference between any of the groups. When comparison is made on the basis of weight of gland per 100 gm. of body weight, the glands of rats in group I (low vitamin B and low iodine) are significantly larger than those of rats in groups IV and VI which were fed vitamin B. The question of whether this was an actual enlargement due to vitamin B deficiency or a relative enlargement due to starvation was answered by paired feeding studies shown in table 3.

They show that starvation rather than a deficiency of vitamin B causes the relative increase in weight of the gland and indicate that in this study comparisons should be made of the actual weight of the glands.

Eighty-two- and 123-day periods. In each of these two periods, there was no significant change from the 41-day period. The actual weight of the glands of rats fed the vitaman B deficient diets remained nearly constant, while the weight of the glands of rats fed the vitamin B containing diets increased slightly with the increase in body weight.

The value for each group, including the stock animal group, is less than 50% of the value given by Donaldson ('24) for normal rats of the same weight.

c. Iodine content and concentration of the glands. The glands of rats in group I had an iodine content of approximately 5.0 γ and a concentration of approximately 0.2%, dry basis, in each of the three periods. This was the lowest concentration found in any of the groups and is more than twice the 0.075% necessary (Remington and Levine, '36) to induce enlargement of the gland. However, it is within the range where hyperplasia should occur in some cases. The average iodine content of the thyroid of rats when weaned was 4.5 γ so that in group I the iodine contents were just maintained. The glands of rats in all of the other groups had higher concentrations of iodine as shown in table 2.

d. Vitamin B and thyroid iodine concentration. With a comparable iodine intake the thyroids of rats fed the diet containing dried yeast (diet 40) had a higher concentration of iodine than those of rats fed the diet containing autoclaved yeast (diet 39A) or the diet containing autoclaved yeast and vitamin B extract (diet 39B). The values in the above order for the 41-day period were 1.5% ± 0.3%, 0.53% ± 0.05%, and 0.36% ± 0.06% iodine calculated on the dry basis. In the 52-day period the values were 0.78% ± 0.12%, 0.47% ± 0.05%, and 0.30% ± 0.01% iodine. The values for the 123-day period were 0.83% ± 0.23% iodine for diet 40 and 0.67% ± 0.04% iodine for diet 39A. Values were not obtained for diet 39B in the 123-day period.

In the 123-day period the difference in iodine concentration of the glands of rats fed diet 40 and those of rats fed diet 39A is not significant. The higher concentration in the glands of rats fed diet 39A may have been caused by the dried yeast that was fed in order to keep them alive for the 123 days. It is shown in table 2 that the growth was practically the same for rats on diet 40 and diet 39B, which indicates that there was not a deficiency of vitamin B in diet 39B and therefore the lower thyroid iodine concentration is not a result of vitamin B deficiency. The results suggest that some factor other than vitamin B is present in dried yeast which affects the thyroid iodine concentration and that this factor is lost when the yeast is autoclaved.

e. Microscopic findings. Stock. The acini of most of the glands contained medium staining colloid and were lined with cuboidal epithelium (fig. 1). Seven glands out of thirty-three showed slight hyperplasia.

Group I. The glands of rats in this group were similar to those of the stock animals (fig. 2). Three out of twenty showed a slight hyperplasia.

Group II. Seventeen of the nineteen glands examined had dilated acini with low cuboidal epithelial lining and dense pink staining colloid filling the lumina. The appearance was that of moderate colloid goiter (fig. 3). The other two glands were slightly hyperplastic.

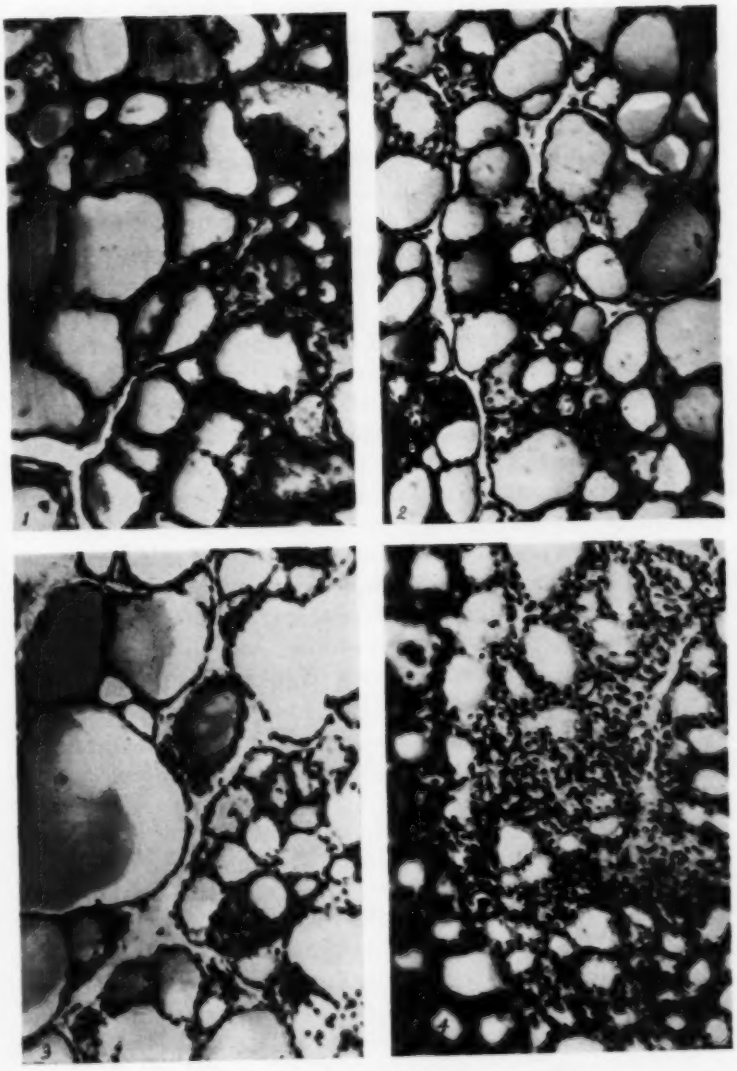
Group III. Eleven glands out of twelve appeared normal, the twelfth being slightly hyperplastic.

Fig. 1 Normal gland of rat from stock colony. The acini are of average size, lined by columnar epithelium and the lumina contain lightly staining colloid. (Age, 110 days.)

Fig. 2 Group I, diet 38 (low iodine, low vitamin B). Numerous small acini lined or filled with cuboidal cells. The larger acini have lumina filled with lightly staining colloid material. (Age, 108 days.)

Fig. 3 Group II, diet 39 (low vitamin B, 0.0038% iodine). Shows large dilated acini filled with darkly staining colloid, also smaller acini lined with cuboidal epithelium. The condition resembles colloid goiter. (Age, 108 days.)

Fig. 4 Group VI, diet 41 (basal diet with vitamin B but no I_2). Small acini filled with columnar cells. The few lumina present are filled with lightly staining colloid. (Age, 108 days.)



Figures 1 to 4

Group IV (diet 40). Twenty-one glands out of twenty-seven had acini filled with dense pink staining colloid and six were slightly hyperplastic.

Diet 39B. Twelve glands out of fourteen were normal; two were slightly hyperplastic.

Group V. The glands in this group were very similar to those in group IV. Five out of eighteen were slightly hyperplastic.

Group VI (diet 39B). Seven glands out of twelve were normal. Five showed varying degrees of hyperplastia, in no case extensive.

Diet 41. Eleven glands out of twenty-four were hyperplastic (fig. 4), with acini varying in size and shape; in many instances the smaller ones were filled with cells. The larger acini were lined with cuboidal or columnar epithelium and contained lightly staining colloid.

f. Condition simulating colloid goiter. Seventeen out of nineteen rats examined in group II, fed the vitamin B deficient diet containing 0.0038% iodine, had thyroid glands which microscopically simulated colloid goiter. Group II was the only group in which this condition occurred. Rats in group I, fed the same diet with no iodine added, had glands similar to those of the stock animals. Rats in group II, fed the same diet with 0.019% iodine, also had normal glands. Rats in group IV, which received vitamin B in addition to the diet used in group II, had thyroids which also resembled those of the stock animals.

The vitamin B was supplied as dried yeast in diet 40 and as an extract of wheat germ in diet 39B (table 1). The additional iodine supplied by these sources of the vitamin was negligible. As near as could be determined by the method used, the iodine content of dried yeast was the same as that of autoclaved yeast. Iodine was determined in the combined diet and extract and gave the same value as the diet alone. Six rats in group IV with food intake limited to the amount eaten by littermates in group II also had normal glands.

Hellwig ('34) has produced colloid goiter consistently in rats by feeding 2 γ potassium iodide daily with a diet that normally produced a parenchymatous type of goiter. With 20 γ of potassium iodide daily, a normal sized gland, microscopically resembling resting colloid goiter seen in human subjects, was obtained. The effect of a higher iodine intake was not determined.

In this experiment goiter was not produced when iodine was not added to the diet. In the light of Hellwig's ('34) experiments, the production of a colloid goiter-like condition in such a large per cent of the rats in group II is therefore surprising and difficult to explain. If there is a relation between the vitamin B requirements and metabolism as has been shown by Cowgill and Palmieri ('33), Hendricks ('33), and others, it is possible that in vitamin B deficiency the demands made on the thyroid are so small that a tendency to goiter under the conditions of this experiment would not show up. Another possible explanation would be that in vitamin B deficiency an intake of iodine greatly in excess of the physiological requirement will cause a condition which microscopically resembles colloid goiter.

Study of the hypophysis

Since a relation is known to exist between the hypophysis and the thyroid, the hypophyses of all animals studied were weighed in the same manner as has been described for the thyroid. In each period, the glands of rats fed diet 41 were the largest, but the individual variation was so great that the enlargement was not significant.

SUMMARY

1. Vitamin B deficiency alone did not affect the size, structure, or iodine content of the thyroid gland of the rat.

2. Rats fed a diet deficient in vitamin B and containing 0.0038% iodine developed a thyroid gland that microscopically simulated colloid goiter. This condition did not develop when the diet contained 0.019% iodine or vitamin B.

3. The results suggest that some factor present in yeast which is lost when the yeast is autoclaved and which is not supplied by a vitamin B containing extract causes an increase in the iodine content and concentration of the thyroid.

4. Differences in the weight of the hypophysis of rats on the different diets were not statistically significant.

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THE COMPOSITION OF MILK FROM STOCK RATS AND AN APPARATUS FOR MILKING SMALL LABORATORY ANIMALS[1]

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TWO TEXT FIGURES AND ONE PLATE (ONE FIGURE)

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In spite of the fact that the rat is widely employed in laboratory investigations, very little is known concerning the composition of its milk. Hatai ('17) obtained milk from the stomachs of suckling young, but apparently in quantities insufficient for complete analysis. More recently, Mayer ('35) has reported the composition of milk obtained by the same method. In spite of these studies, it must be noted that the mixing of milk with salivary and gastric juices, even for the shortest possible time, may result in considerable change in composition.

We have been successful in milking rats directly. More than 600 cc. of milk from stock rats has thus been made available, and in the present communication we report the complete analysis of milk from stock rats.

METHODS AND RESULTS

Because of the limited amount of milk that may be obtained from an individual rat, pooled samples were used for all analyses. We first determined the changes in composition at various stages of lactation; arbitrarily, at the eighth, thirteenth, eighteenth and twenty-fourth days of lactation. The

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results are reported in table 1. Total solids were determined by heating a weighed sample at the temperature of boiling water until almost dry, and then placing in a vacuum oven at 75°C. for 4 hours; total ash, by heating an evaporated, weighed sample at 550°C. in an electric muffle; total nitrogen, by the standard Kjeldahl with selenium added to speed digestion (Lauro, '31); fat, by extraction with alcohol and ether in a Mojonnier and Troy ('25) apparatus, and lactose, by iodometric titration of the oxidized Cu_2O (Methods of Association of Official Agricultural Chemists, '30 (a)) using the

TABLE 1

Composition of rat milk at various stages of lactation

	DAY OF LACTATION				
	8th	13th	18th	24th	Average
Specific gravity $\frac{25}{25}$	1.049	1.044	1.046	1.047	1.047
Solids (as determined), %	31.02	32.87	27.13	35.86	31.7
Total nitrogen × 6.38	11.81	11.71	11.70	(11.85)	11.8
Fat	14.03	16.05	12.14	16.93	14.8
Carbohydrate	2.40	3.05	3.40	2.46	2.8
Ash	1.70	1.48	(1.50)	(1.36)	1.5
Solids (sum)	30.0	32.3	28.7	32.6	30.9

Parentheses indicate that more than one pooled sample was used in obtaining an average value.

filtrate from the differential precipitation of casein and lactalbumin (Methods of Association of Official Agricultural Chemists, '30 (b)) after fat extraction.[2]

In spite of the fact that, in the early stages of lactation, adequate milk for analysis was obtained only with difficulty (raising the question of evaporation), no great differences in composition were noted. This may be more readily understood if it is remembered that 1 day in the life of the rat is equivalent to 1 month of human life (Donaldson, '24). Eight days after parturition, lactation in the rat, therefore, is rather completely established. At the period of greatest milk production (eighteenth day after parturition), total solids are

[2] A known amount of lactose was added to a sample of rat milk. After performing the indicated separations, the lactose was quantitatively recovered.

slightly less than at other periods—reflecting a slight drop in fat content.

In view of the high nitrogen content of rat milk, a study of the nitrogen distribution was considered desirable. A condensed summary of such studies is given in table 2, and it is to be noted that the percentage distribution of the nitrogenous constituents does not differ greatly from that recorded for

TABLE 2

Protein distribution in pooled samples of rat milk. (The prime figures indicate the number of determinations)

SAMPLE NO.	VOLUME OF SAMPLE	TOTAL, N × 6.38 [a]	TOTAL PRECIPITATED PROTEIN [b]	CASEIN (METHODS, A.O.A.C., '30) [b]	ALBUMIN	GLOBULIN (MOIR, '31)	N.P.N. [d]	ALBUMIN + GLOBULIN [c]	CASEIN + GLOBULIN (MOIR, '31)
	cc.	%	%	%	%	%	%	%	%
1	29	9.57[3]	89.2[3]	79.8[3]	6.11[3]		13.6[4]	13.3[1]	
2	38	10.68[3]	89.0[4]	78.7[3]	7.49[3]		11.8[3]	12.4[3]	
3	45	10.66[12]	89.3[3]	78.2[6]	9.85[3]		11.2[7]	12.7[3]	
4	49	10.22[12]	89.4[3]	76.9[4]	7.60[4]	4.8[4]	11.2[6]	13.4[3]	82.8[3]
Average	..	10.28[6]	89.2	78.2	7.76	4.8	11.9	12.9	82.8
Cow (Davies, '32)	..	3.17	94.1	76.1	12.6	5.4	5.9	18.0	...

[a] Average of N determinations on whole milk and of the sum of the separate nitrogenous constituents in each complete set of partitions, × 6.38.

[b] Average of total protein precipitated by trichloracetic acid, and the sum of the individually partitioned protein precipitates in each complete set of observations.

[c] Precipitated with trichloracetic acid after separation of casein.

[d] Expressed in terms of percentage nitrogen × 6.38.

[e] Analyses made in late spring and early summer. The effect of season may account for the difference between this figure and those reported in tables 1 and 3.

cow's milk. Casein and albumin were determined by the Methods of the Association of Official Agricultural Chemists ('30 (b)), save that in the analysis of the fourth sample, these methods were supplemented by the procedures of Moir ('31). Non-protein nitrogen was determined on the filtrates only after the addition of trichloracetic acid.

In table 2 it will be noted that the percentage of total nitrogen as casein, and as globulin, in cow's and rat's milk, is approximately equal; but that the percentage of total nitrogen as albumin is less in the latter in spite of a larger absolute amount (table 3). Conversely, non-protein nitrogen ($\times$ 6.38) is greater when expressed as percentage in milk—or as percentage total nitrogen, in rat's milk than in cow's milk.

The composition of rat milk, as compared with that of cow and human milk, is given in table 3. The percentages saturated and unsaturated fatty acids were determined by the lead salt ether method (Jamieson, '32 (a)), iodine number by the Wijs method (Jamieson, '32 (b)), and saponification number in the usual way (Jamieson, '32 (c)). The Association of Official Agricultural Chemists procedures were followed for the Reichert-Meissl and Polenske values (Methods, Association of Official Agricultural Chemists, '30 (c)). Calcium in the ash was determined by the micro method of Tisdall and Kramer (Peters and Van Slyke, '32 (a)); magnesium by the 8-hydroxy quinoline method of Greenberg and Mackey ('32), sodium as the uranyl zinc sodium acetate (Butler and Tuthill's modification of Barber and Kolthoff's method (Peters and Van Slyke, '32 (b)), and potassium by difference, after determination of total alkali sulphates (Peters and Van Slyke, '32 (c)). Total phosphorus was determined colorimetrically after precipitation with strychnine molybdate, according to Tisdall (Peters and Van Slyke, '32 (d)); chloride by determination of silver nitrate required for complete precipitation (Peters and Van Slyke, '32 (e)). Iron was determined by the method of Winter ('31), and copper by the method of Supplee and Bellis ('22).

The tenfold concentration of iron and copper in rat's milk, as compared with cow's milk and human milk, is worthy of note. Similarly, calcium, phosphorus, and magnesium are present in three times the concentration that they are in cow's milk; whereas, sodium, potassium and chlorine are present in amounts strictly comparable with that present in cow's milk.

TABLE 3

Comparison of the composition of rat, cow and human milk

		RAT MILK		COW MILK		HUMAN MILK	
Specific gravity		1.047		1.031[1]		1.031[1]	
Protein, %		11.77		3.42[2]		1.25[1]	
Lactalbumin	Cale'd,		0.91		0.56[2]		0.75[2]
Casein	table		9.20		2.86[2]		0.50[2]
N. P. Nitrogen × 638	3		1.33		0.20[11]		....
Carbohydrate, %		2.83		4.75[1]		7.50	
Fat, %		14.79		3.50[1]		3.50[1]	
% saturated acids			46.5[3]		67.4[4]		43.5[7]
% unsaturated acids			48.4		27.4[4]		55.1[7]
Av. mol. weight saturated acids			248.2		....		258.0[7]
Av. mol. weight unsaturated acids			267.8		....		.....
Saponification number			231.0		230.5[3]		205.1[3]
Iodine number			41.0		32.0[3]		56.2[3]
Reichert-Meissl number			4.9		28.0[3]		2.5[3]
Polenske number			11.7		2.3[3]		0.1[3]
Refractive index			1.461[10,7]		1.455[10,2]		1.457[10,7]
Ash		1.50		0.75[1]		0.20[1]	
Potassium			0.170		0.154[1]		0.048[1]
Sodium			0.076		0.061[1]		0.011[1]
Chloride			0.117		0.116[1]		0.036[1]
Calcium			0.349		0.122[1]		0.034[1]
Phosphorus			0.272		0.090[1]		0.015[1]
Magnesium			0.031		0.013[1]		0.005[1]
Iron			0.0007[5]		0.00007[1,5]		0.0002[1,5]
Copper			0.0007[5]		0.00006[5]		0.00005[10]
Ca/P ratio			1.28		1.35		2.26
Total solids (sum)		30.89		12.42		12.45	
Total solids (determined)		31.7		....		....	
pH		6.6		6.7[1]		7.0[1]	
Buffer capacity[8]		109.0		42.0		13.0	
Lysozyme content		Rat milk contains more than cow milk, less than human milk[6]					
% calories, as protein		24.6		21.7		7.5	
% calories, as fat		69.5		48.8		47.4	
% calories, as carbohydrate		5.9		29.5		45.1	

[1] Holt, L. E., Jr. and R. McIntosh, Holt's Diseases of Infancy and Childhood, 10th ed., p. 147. New York.

[2] Associates of Rogers, Fundamentals of Dairy Science, pp. 24 and 80. New York.

[3] Exclusive of acids represented by R-M and Polenske numbers.

[4] Holland, P. B. and J. P. Buckley, J. Agri. Res., vol. 12, p. 719, 1918; from Lewkowitsch, vol. 2, p. 820, 6th ed. London.

[5] We are indebted to Messrs. Raymond Barton and Norman J. Miller of this laboratory for the Fe and Cu determinations, and lysozyme studies, respectively. Regarding lysozyme in various milks compare Prickett, P. S., N. J. Miller and F. G. McDonald., J. Bact., vol. 25, p. 61, 1933.

[6] Bosworth, A. W., J. Biol. Chem., vol. 106, p. 235, 1934.

[7] Roller, P. E., J. Pediatrics, vol. 4, p. 238, 1934.

[8] When defined as the number of cubic centimeters of 0.1 N HCl to give a pH of 4.7 (the iso-electric point of casein) using 100 cc. of milk.

[9] Davies, W. L., The Chemistry of Milk, p. 19, 1936. Van Nostrand, New York.

[10] Hess, A. F., G. C. Supplee and B. Bellis, J. Biol. Chem., vol. 57, p. 725, 1923.

[11] Davies, W. L., J. Dairy Res., vol. 4, p. 142, 1932.

We question whether these differences are suggestive in relation to the physiological requirements for rapid growth and the fact that rats are cartilagenous at birth.

In view of the fact that our stock diet contained 65% of the fat as butter fat, the difference in the Reichert-Meissl and Polenske numbers of butter fat as compared with the fat of rat's milk should not pass unnoticed. In partial explanation, at least two factors influence slightly the composition of milk fat—the season of the year, and the food fed. Changes in composition are noted primarily in the degree of unsaturation and the percentage volatile acids (Davies, '36 (a)). Ingested volatile fatty acids, below lauric, are presumably metabolized by the animal and are not manifested by significantly increased Reichert-Meissl or Polenske values (Hilditch and Sleightholme, '30). To a large extent, therefore, the percentage volatile acids in a milk may be regarded as characteristic of the species.

In planning experimental diets, it is of some theoretical interest to know the distribution of calories ordinarily ingested by the suckling. It will be noted (table 3) that the diet of the young rat contains some 69% of the total calories as fat, and but 5.9% as carbohydrate. In the usual experimental diet, a large percentage of the energy requirement is supplied by carbohydrate.

Buffer curves of rat, cow, human, and guinea pig milk before and after dilution were determined by means of the micro-quinhydrone electrode (Cullen, '29) using a type K, Leeds and Northrup potentiometer. The resulting curves are given in figure 1. The high buffer quality of rat's milk is but another way of expressing the degree of concentration of milk protein and milk salts.

SUMMARY

1. More than 600 cc. of milk from stock rats, obtained by means of a micro-milking device, have been analyzed. Milk solids are approximately three times as concentrated as in cow's milk. Compared to human and cow's milk, rat milk is low in carbohydrate, and high in protein and fat.

2. The average composition of rat milk obtained at various stages of lactation is: total solids, 31.7%; protein, 11.8%; fat, 14.8%; carbohydrate, 2.8%; and ash, 1.5%.

3. Studies on the distribution of nitrogen in the total protein, on the constants of the extracted butter fat, and on the composition of the ash of rat's milk are reported.

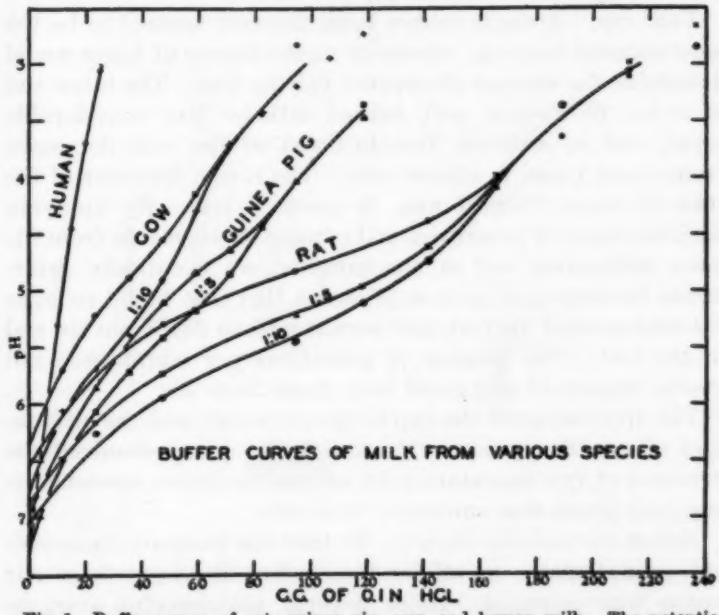

Fig. 1 Buffer curves of rat, guinea pig, cow, and human milk. The potentiometric determinations were made on diluted and undiluted samples. The ratios (1: 10, etc.) indicate the degree dilution; the acid was equally diluted in each case.

4. The buffer values of rat and guinea pig milk have been determined, and are compared with similar values for cow's milk and human milk.

ADDENDUM

With the thought that other investigators may wish to study the effect of drugs, hormones, surgical procedures or dietary

changes on the composition of rat milk, we give, in the following brief paragraphs, a description of our technic.

In milking, at least three factors must be considered: 1) the support of the small, collapsible teat, 2) the negative pressure applied to the end of the teat (which, if excessive, may cause congestion), and 3) the number of alternating pulsations (suction applied and released) per minute.

Teat cup. A small rubber tube, tapered, seemed to be the most suitable teat cup, especially as the degree of taper would determine the amount of support for the teat. The large end of a no. 10 French, soft rubber catheter has considerable taper, and in addition, the thickness of the wall decreases from about 1 mm. to almost zero. The inside diameter of the tube increases from 1 mm. to about 6 mm. By applying various negative pressures, and cutting off segments from the more collapsible end of the catheter, we eventually determined the degree of suction (7 inches Hg) that would collapse the tube around the teat, and permit milk to flow from the end of the teat. The number of pulsations per minute was not overly important and could vary from 25 to 40.

The application of the cup to the rat's teat, and the alternation of negative pressure from a water pump required the presence of two operators. An automatic device, operable by one individual, was eminently desirable.

Automatic milking device. To take the place of the assistant, an automatic cut-off for the suction line from the water pump was essential. After several unsuccessful arrangements a magnetic switch (220 volts A.C.) (after removal of all accessories so as to leave only the electro-magnet and the movable arm), proved satisfactory. A copper wire, attached to the movable arm, was passed over a piece of flexible rubber tubing (connected as a by-pass to the water pump and teat cup) held firmly between two segments of stationary glass tubing. When the switch is energized, the arm moves downward, crimps the rubber tubing and prevents inlet of air. Release of the arm permits the rubber tube to regain its original shape and admit air. The switch is enclosed in a wooden box to reduce noise.

Alternating pulsations were obtained by connecting a mercury U-tube (one arm sealed) with a suitable relay (20,000 ohms), and the magnetic switch. In order to allow adequate lag in pulsation (a drop from 7 inches negative pressure to zero pressure, before the switch was re-energized) two adjustable electrodes were placed at different heights in one arm of the U-tube. By sticking a small nail through a cork,

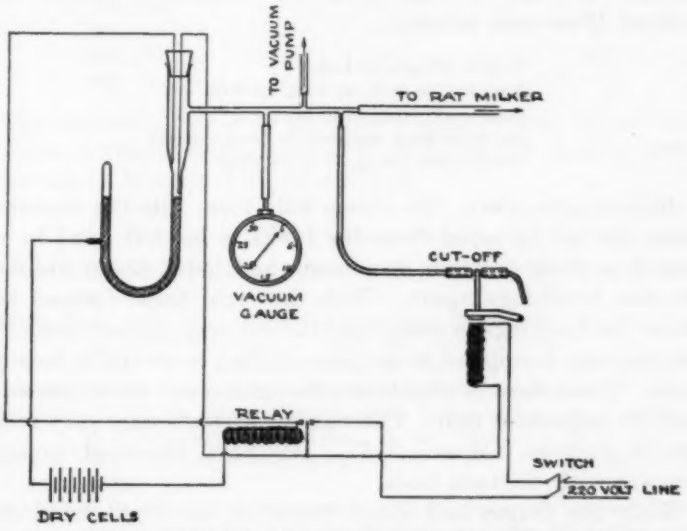

Fig. 2 Diagram of electrical connections for rat milking apparatus

the null-point of the relay became a contact point.[3] The electrical connections are shown in figure 2. When the mercury made contact with the higher electrode, the magnetic switch was de-energized, and it was not re-energized until the mercury had fallen below the lower electrode.

Suction was obtained from an ordinary water pump, but a vacuum line serves even better as a source of negative pressure.

[3] We are indebted to Mr. Lee Stone of Mead Johnson & Company for assistance in the electrical design.

A picture of the machine is given in plate 1. The apparatus may be successfully used in milking rats and guinea pigs. We have not used it on other laboratory animals.

Preparation of rats. Obviously, milk must be in the glands before any can be obtained. The amount of milk available varies with many factors, and we list in the table below those conditions which are preferred in order to obtain the largest possible amount of milk from our laboratory stock rats (inbred Wisconsin strain):

Weight, 300 gm. or better
Reproductive cycle, at least the third
Number of young, 8 to 10
Day of milking, sixteenth to twenty-second
Absent from young, 12 to 16 hours

Milking procedure. To obtain milk from rats the operator holds the rat (grasped from the back) in his left hand in an almost vertical position, the thumb and index finger holding the two front legs apart. With the right hand (placed between the hind legs in order that the rat may support herself) the teat cup is applied to any one of the six centrally located teats. Those most available are the second and third thoracic, and the abdominal pair. The number of teats may vary from ten to thirteen (Myers, '16). We have observed several females with thirteen teats.

With the thumb and index finger of the right hand the operator gently strokes the breasts, in order to work the milk toward the teat. Slight pressure is exerted with each development of negative pressure by the machine. The perfection of this procedure is one of the principal essentials for successful milking.

Amount of milk obtained. The mother rat is removed from her young on the evening prior to milking. The next morning her breasts are usually filled with milk. We have not been successful in obtaining milk, night and morning, every day. One milking on two alternate mornings at the height of lactation is about all that can be expected, if one is not satisfied with small volumes. From about 10% of stock rats no milk at all was obtained.

The volume of milk varies with the stage of lactation, as indicated in the following table. This parallels the increasing amount of milk obtainable from the stomachs of nursing young (Mayer, '35).

Volume of milk obtainable at different stages of lactation

Day of lactation	Average volume per rat cc.	Maximum volume from a single rat cc.
7th– 9th	0.5	3.0
12th–14th	0.8	5.0
16th–18th	1.6	8.0
24th	0.7	5.0

By excluding rats with no milk in their breasts the average volume obtained at the sixteenth to eighteenth days of lactation can be increased to about 2.1 cc.

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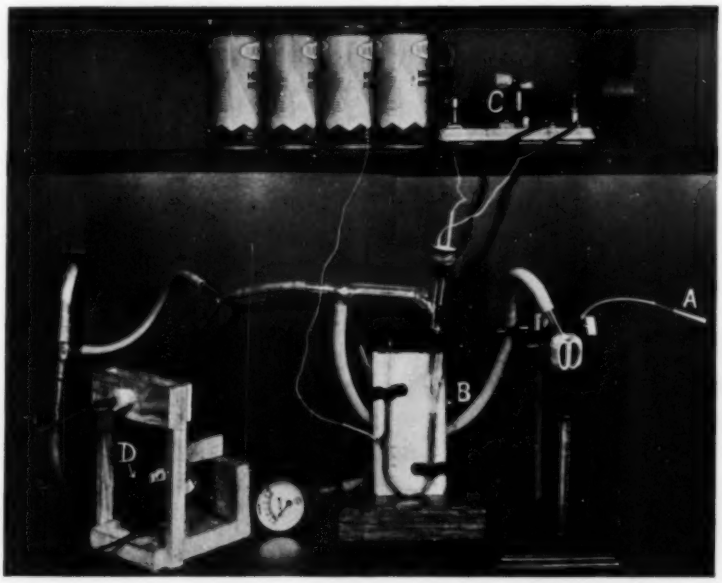

Photograph of rat milking machine. A, teat cup; B, mercury U-tube with two electrodes at different heights in one arm; C, 20,000 ohm relay; D, magnetic switch.

A CONSIDERATION OF THE NUTRITIVE STATE IN THE METABOLISM OF WOMEN DURING PREGNANCY [1]

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THREE FIGURES

(Received for publication September 8, 1936)

Significant individual differences have been pointed out in the metabolic processes of healthy women (Macy, Hunscher, Nims and McCosh, '30; Hunscher, Donelson, Erickson and Macy, '34) and children (Hunscher, Cope, Noll, Macy, Cooley, Penberthy and Armstrong, '32; Hunscher, Cope, Noll and Macy, '33; Macy, Hunscher, Hummel, Bates and Poole, '36) as shown by the rate of retention of the acid-base mineral elements and nitrogen when the subjects were observed under similar dietary and environmental conditions. In view of these findings it becomes increasingly difficult to compare the metabolic responses of several individuals (Hunscher, Hummel, Erickson and Macy, '35) under the demands of preg-

[1] A preliminary report of this paper was presented before the Division of Biological Chemistry at the ninety-second meeting of the American Chemical Society in Pittsburgh, Pa., on September 7, 1936. Other papers on the basal metabolic rate and the metabolism balances during delivery, puerperium and lactation will be reported later.

nancy without knowing something of the underlying factors governing them such as nutritive state and other physiological conditions.

Although Rubner so long ago showed that the nutritive state of the individual markedly influenced the rate of storage in the body, little attention has been given to it in past interpretations of metabolic balance data in determining dietary requirements of man and animal. Of the same diet more was retained when the nitrogen content of the dog was low than when it was high. In interpreting Rubner's findings, Lusk ('21) states that

> According to these laws adult cells which have been depleted of their protein may gradually improve their nutritive condition until they reach an optimum, at which point they lose their power to attach additional protein.

In the case of calcium, phosphorus and iron there is a provision in some of the tissues for an inert deposit or bodily reserve (Bauer, Aub and Albright, '29) in contrast to nitrogen which has become an integral part of soft tissues of the body without extensive deposit. Fairbanks and Mitchell ('36) have recently shown in experiments with rats that

> Differences in the degree of saturation of the skeletal tissues with respect to calcium, brought about by previous subsistence upon diets differing in their contents of this element produce inequalities in the retention of calcium under uniform conditions of calcium nutrition such that low saturation is associated with subsequent high retention of calcium. It follows that the rate of calcium retention by growing animals under conditions of adequate nutrition measures the requirement of calcium only when the calcium stores have been saturated by appropriate pre-feeding. Otherwise the observed calcium retentions will be greater than the day to day requirements of calcium.

Realizing the necessity for long time observations and appreciating the importance of previous nutritional state in metabolic balance studies, Clark ('26) pioneered the field in a most admirable manner. The success of his endeavors culmi-

nated in the demonstration of the effect of unfilled or depleted body reserves of five men who under strict metabolic regime stored during 28 continuous weeks nitrogen and the acid-base mineral elements in amounts characteristic of their bodies' former nutritive deprivations. With the calcium intake of approximately 0.90 gm. per day the per cent of the intake retained for the men were in the following ascending order: 3.1, 6.5, 17.2 and 30.6—findings which were commensurate with those predicted by Clark from his knowledge of the dietary habits of the men during previous years. Likewise, other recognized differences in human metabolic response decisively due to the nutritive state have been shown in undernourished children by Stearns ('31), Wang, Kaucher and Frank ('28) and Wang, Kern and Kaucher ('29) as well as by Daniels, Hutton, Knott, Wright and Forman ('35).

The present report records the metabolic balances of an 18-year-old primapara who was observed uninterruptedly during the final 65 days of gestation. Her medical history showed an unsatisfactory nutritional background for the preceding 6 years. For the purpose of determining if the nutritive state of the maternal organism is one of the causative factors for the differences observed among individuals under the physiologic influences imposed by pregnancy (Hunscher, Hummel, Erickson and Macy, '35; Hummel, Sternberger, Hunscher and Macy, '36) the data obtained have been compared with similar ones collected continuously to term on two healthy women during the same interval of reproduction. It is hoped that these data will contribute to the interpretation of the metabolic balance method for determining dietary requirements for pregnancy.

EXPERIMENTAL PROCEDURE

Subject. An 18-year-old girl of American descent who had been under the care of the staff in the Department of Pediatrics of the Henry Ford Hospital, Detroit, Michigan, became available for metabolic observations during the last 65 days

of her first pregnancy.[2] She was maintained under highly controlled conditions of study in a private room in the hospital under the constant supervision of specific doctors, nurses and dietitians.[3] Being a ward of a child caring institution all of her life her food consumption and condition of work were not always optimal for a growing adolescent, and she was known to be slightly underweight reaching a pre-gravid body weight of approximately 48 kg. and a height of 158 cm.

During the study the subject appeared happy and serene and seemed to have a feeling of security. She was intelligent and alert in following directions concerning the consumption of food and fluids, as well as the collection of the urine and separation of it from the feces. There was genuine interest and desire on the part of the subject in making all observations successful.

Dietary. A series of diets was planned for 10 consecutive days so that the daily food intake was fairly constant in composition yet by substituting the usual common fruits, vegetables, cereals and meat in food equivalents not only interesting

[2] Case history. I.M.B. was first seen at the age of 12 in 1929 with a history of exposure to a mother who died of tuberculosis. She showed at that time some malnutrition, chronically infected tonsils, a positive reaction to tuberculin but a negative chest plate. Her weight at that time was 84.5 pounds with an estimated weight for height and age according to the Baldwin Wood tables of 95. Tonsils were removed and she was placed in a boarding home. Followed twice a year since then, her reactivity to tuberculin persisted but ten roentgenograms of the chest at intervals through that period have been negative. Her weight curve during that time has been as follows:

Date	*Weight*	*Estimated weight*
8/12/29	84.5	95
10/25/30	90½	102
8/ 9/32	109	113
7/11/33	106	115
7/16/34	107½	117
12/26/34	103½	117

At the beginning of the study, the urine and blood findings were not abnormal. The basal metabolic rate was —3 with an RQ of 0.79. The NPN was 23.5 mg. per 100 cc. of blood. Examination since the study was completed revealed no tuberculosis by x-ray.

[3] We desire to acknowledge gratefully the assistance of the following: Dr. Frank Poole supervised the collections; Dr. Frank Hartman and Miss Spangler were responsible for the metabolism determinations; Miss Foster and Miss Smithyman were in charge of the diets; Miss MacLaren controlled the activity of the patient throughout her stay, and Drs. J. P. Pratt and Stolpman supervised the obstetrical care of the patient.

but varied and appetizing menus were possible. The set of diets was rotated every 10 days to the complete satisfaction of the subject in spite of the regulated food intake. In addition, 1500 gm. of milk containing 125 U.S.P. units of vitamin D per quart and 20 gm. of cheese, the diet not only contained an abundance of all known nutritive principles but furnished approximately 2500 calories per day. A period of 2 weeks was given for adjustment to the diet and to the metabolic routine.

Procedure. The general metabolic procedure and methods of analyses as previously reported by Macy et al. ('30), Hunscher et al. ('35), and Hummel et al. ('36) have been followed. A special dietitian had charge of preparing and weighing of food served to the subject and of samples for analyses.

RESULTS AND DISCUSSION

Table 1 gives a summary of the metabolic data secured during the continuous study of thirteen successive 5-day balances made during the last 65 days of pregnancy. It shows the average daily ingestion, excretion in both the urine and feces, and the resultant maternal gain or loss of calcium, magnesium, sodium, potassium, phosphorus, sulfur, chlorine and nitrogen.

Individual trends in the daily storage of nitrogen and calcium are shown in figure 1 for the primapara (I.M.B.) and the quadripara (L.R.). With a mean intake of 261 mg. of nitrogen per kilogram of body weight the former retained 24.5 mg. while in contrast, the latter with a lower intake stored 38.5 mg. per day. In terms of total nitrogen exchange, the mean daily intake of I.M.B. and L.R. were 14.05 gm. (13.51 to 14.82 gm.) and 19.03 gm. (18.34 to 21.10 gm.) with a mean net daily storage of 1.32 gm. (0.32 to 2.22 gm.) and 3.02 gm. (1.64 to 5.44 gm.), respectively. It is of interest to compare these findings with the other similar studies on women found in the literature, namely, the classic case of Hoffström ('10) and that of Coons and Coons ('35), the latter being carried

TABLE 1

Nitrogen and acid-base mineral elements retained per day by a primapara (I.M.B.) who was studied uninterruptedly during the last 65 days of pregnancy

DAYS ANTE-PARTUM	BODY WEIGHT	NITROGEN				PHOSPHORUS				CHLORINE				SULFUR			
		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
65–69	52.0	14.82	12.46	1.18	1.18									1.16	0.84	0.14	0.32
60–64	52.1	14.28	11.72	1.09	1.47	2.09	1.28	0.64	0.17	4.59	4.64	0.05	—0.10	1.16	0.69	0.14	0.34
54–59	52.3	13.67	11.92	0.98	0.77	1.77	1.20	0.49	0.09	3.85	4.59	0.04	—0.78	0.94	0.67	0.09	0.17
50–53	52.6	14.02	10.96	1.10	1.96	1.90	1.28	0.50	0.12	4.25	3.86	0.05	0.34	1.04	0.63	0.10	0.31
45–49	52.8	13.96	11.80	0.95	1.21	1.83	1.14	0.40	0.29	4.26	4.71	0.02	—0.47	1.03	0.65	0.11	0.28
40–44	53.0	13.99	11.75	1.04	1.20	1.87	1.17	0.46	0.24	4.33	4.06	0.03	0.24	1.02	0.62	0.11	0.28
35–39	53.5	13.95	11.62	0.95	1.38	1.99	1.19	0.42	0.38	4.18	3.95	0.02	0.21	1.06	0.65	0.10	0.30
30–34	54.1	13.86	12.53	1.01	0.32	1.97	1.15	0.53	0.29	4.10	4.60	0.02	—0.52	1.09	0.66	0.14	0.29
25–29	54.4	14.13	11.21	0.70	2.22	1.95	1.04	0.39	0.52	4.15	3.99	0.04	0.12	1.06	0.63	0.11	0.32
20–24	54.9	13.51	10.95	1.02	1.54	2.01	1.11	0.57	0.33	4.22	4.26	0.04	—0.09	0.98	0.64	0.13	0.20
14–19	55.6	14.20	12.16	1.20	0.84	1.97	1.09	0.55	0.33	4.10	4.77	0.06	—0.73	1.08	0.64	0.17	0.28
10–13	56.0	14.19	11.29	1.15	1.75	1.98	1.22	0.54	0.22	4.32	3.88	0.06	0.38	1.02	0.66	0.16	0.20
5– 9	56.7	14.17	11.30	1.08	1.79	1.90	1.20	0.45	0.25	4.42	4.80	0.03	—0.41	1.02	0.67	0.12	0.22
Total gain, 65 days					**86.05**				**16.23**				**—11.28**				**17.49**
Mean per day		**14.05**			**1.32**	**1.93**			**0.27**	**3.91**			**—0.17**	**1.05**			**0.27**

TABLE 1—*continued*

DAYS ANTE-PARTUM	BODY WEIGHT	CALCIUM				MAGNESIUM				SODIUM				POTASSIUM			
		Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance	Intake	Urine	Feces	Balance
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
65–69	52.0	1.96	0.49	0.55	0.93	0.43	0.14	0.22	0.07	2.92	2.86	0.02	0.04	4.05	4.04	0.35	—0.34
60–64	52.1	1.95	0.48	0.84	0.62	0.39	0.10	0.21	0.08	2.70	2.69	0.01	0.00	4.05	3.83	0.34	0.12
54–59	52.3	1.93	0.51	1.07	0.35	0.40	0.13	0.24	0.03	2.71	2.65	nil	0.06	4.05	3.76	0.30	—0.01
50–53	52.6	1.91	0.44	0.89	0.58	0.39	0.11	0.23	0.05	2.84	2.38	nil	0.46	4.05	3.70	0.35	0.00
45–49	52.8	1.91	0.44	0.61	0.86	0.39	0.06	0.20	0.13	2.75	2.78	nil	—0.03	4.05	3.64	0.31	0.10
40–44	53.0	1.97	0.46	0.80	0.70	0.40	0.12	0.19	0.10	2.71	2.49	nil	0.22	4.05	3.90	0.34	—0.19
35–39	53.5	1.94	0.45	0.86	0.63	0.40	0.14	0.20	0.07	2.75	2.46	nil	0.29	4.05	3.74	0.29	0.02
30–34	54.1	1.98	0.44	0.71	0.84	0.38	0.14	0.18	0.05	2.79	2.88	nil	—0.09	4.05	3.64	0.32	0.09
25–29	54.4	1.98	0.38	0.41	1.18	0.39	0.09	0.14	0.16	2.71	2.48	nil	0.23	4.05	3.50	0.30	0.25
20–24	54.9	1.96	0.41	0.51	1.04	0.38	0.18	0.17	0.02	2.82	2.47	nil	0.36	4.05	3.71	0.34	0.00
14–19	55.6	1.96	0.37	1.04	0.55	0.38	0.17	0.20	0.01	2.79	2.96	nil	—0.17	4.05	3.75	0.36	—0.06
10–13	56.0	1.94	0.31	1.25	0.39	0.39	0.15	0.20	0.04	2.79	2.27	nil	0.52	4.05	3.64	0.34	0.07
5– 9	56.7	1.94	0.26	1.07	0.61	0.35	0.16	0.19	0.03	2.77	2.99	nil	—0.22	4.05	3.06	0.34	0.05
Total gain, 65 days					**46.29**				**4.15**				**7.26**				**0.54**
Mean per day		**1.95**			**0.71**	**0.39**			**0.06**	**2.77**			**0.11**	**4.05**			**0.01**

from the thirty-first to thirty-fifth weeks of pregnancy only thus omitting the final interval to term. The total mean daily retention of nitrogen for the former was 1.74 gm. and that for the latter was 2.24 gm.

Hoffström ('10) was the first to show by means of continuous observations on a woman from the seventeenth week of

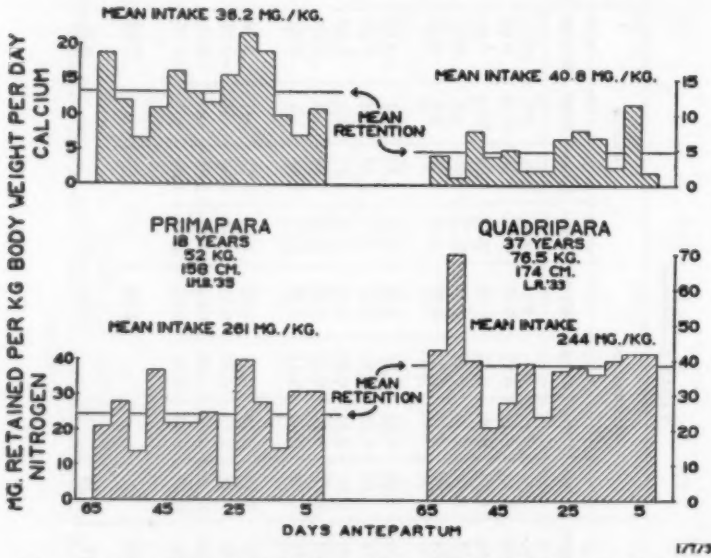

Figure 1 shows the contrast in the continuous daily retentions of calcium and nitrogen per kilogram body weight during 65 days antepartum in two women of widely different backgrounds of nutritional state and physiological constitution, that of the primipara (I.M.B.) being unsatisfactory and that of the quadripara (L.R.) satisfactory.

pregnancy to term that the maternal body acquired a reserve of stored elements beyond the estimated needs of her preparation for puerperium, lactation and the development of the fetus. In accordance with the weight of evidence on the influence of nitrogen retention in pregnancy to milk secretion as shown in the recent review by Garry and Stivens ('36), L.R., who was capable of secreting a large volume of milk beyond

that necessary to nourish her own baby, stored a total of 196.25 gm. of nitrogen during the final 65 days of pregnancy. On the other hand, I.M.B., who developed only an average milk supply, stored but 86.05 gm. of nitrogen, while the case of Hoffström stored 109.8 gm. during the same interval of time. Figure 2 shows that the fetal needs were well covered

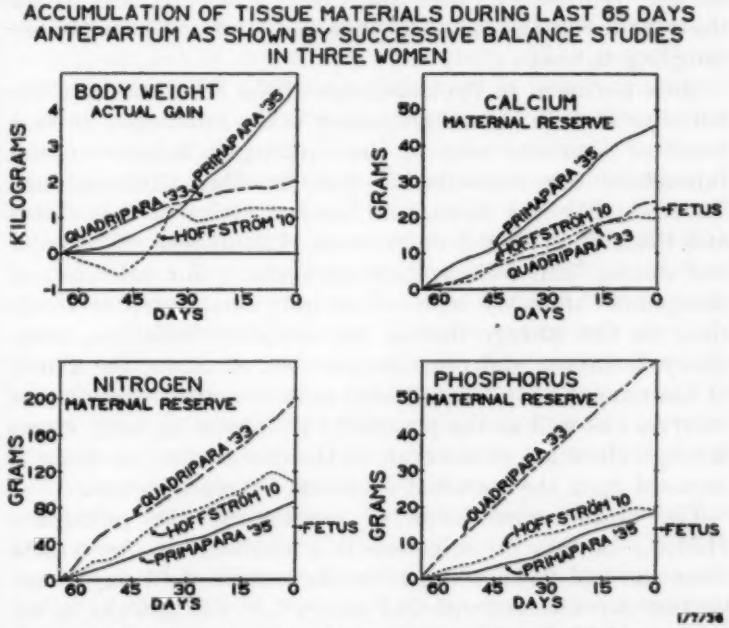

Figure 2 illustrates the accumulation of tissue materials during the last 65 days of gestation as shown by successive balance studies on three women, i.e., Hoffström's ('10) case, the quadripara studied in 1933 and the primapara of the present study as determined by continuous observations during the same interval of time.

by the maternal acquisition of nitrogen during only the last trimester of gestation in all three cases, and that the phosphorus storage was in accord with the nitrogen.

In marked contrast to the results on nitrogen storage are these on calcium (table 1 and fig. 1). With an intake of 32.2

mg. of calcium per kilogram body weight per day, I.M.B. retained 13.2 mg., whereas with an intake of 40.8 mg., L.R. stored on the average only 4.8 mg. daily per kilogram body weight. The total daily retention of 0.71 gm. of calcium per day by I.M.B. exceeded by far the 0.38 gm. by L.R., 0.32 gm. by the woman reported by Hoffström ('10) and 0.28 gm. by the one recorded by Coons and Coons ('35) and falls within the wide variations found by Pyle and Huff ('36) on 24-hour sampling balances.

More pertinent to the knowledge of the relationship of the nutritive state in human pregnancy is the cumulative storage trend of elements seen in the continuous balance studies throughout one reproductive cycle in dogs (Toverud and Toverud, '31) and throughout several cycles in rats (Goss and Schmidt, '30) and dairy cows (Ellenberger, Newlander and Jones, '32). These data particularly for calcium and phosphorus show the influence of individual nutritive conditions on the storage during one complete cycle, i.e., pregnancy, lactation, and reproductive rest, or more; the ability of the animal body to accelerate retention after depletion of reserves; as well as the possibility of increasing body stores through elevation of minerals in the diet so that no drain is imposed upon the maternal organism by reproduction.

The large accumulation of calcium by the primapara (I.M.B.) of this investigation is a challenging observation since she laid down 46.3 gm. in the period of 65 days antepartum as contrasted with 24.7 gm. by L.R. and 20.0 gm. by the subject of Hoffström. It is evident that the three women were under considerably different physiological influences in addition to gravidity (fig. 2) for the former acquired more than twice the actual calcium needs of the fetus during the final thirteen 5-day periods of pregnancy, whereas the other two cases had just satisfied the average fetal requirements during the same interval.

The total accumulation for the nutritive processes of the adjusting maternal and growing fetal bodies is a matter of greatest importance since it may include

the filling in of the nutritive reserves that perchance were existent through a previous state of undernutrition and in some cases upon the growth of the maternal body itself (Hunscher, Hummel, Erickson and Macy, '35).

The dire results of extremely calcium impoverished women are seen in osteomalacia. Maxwell and co-workers ('25, '35) along with Miles and Feng ('25) have studied the etiology, the metabolism and treatment of this deficiency disease, as it is intensified by reproductive demands. They have shown that the bones of adolescent girls may be impoverished by lack of calcium, of vitamin D, or both, and that severe crippling results during reproduction and particularly with repeated cycles. Not only the maternal bones are drained upon but the fetus, as resistant as it is in intra-uterine development to nutritive inadequacies, shows evidence of rickets and osteoporosis of the bones and gross hypoplasia of the enamel of the teeth (Wolfe, '35; Maxwell, '35). Thus in human pregnancy, as in animal (Davidson, '30), the young may start life with a reduced store of calcium. In such a case the individual is forced to carry a greater metabolic load than it is physiologically capable of managing, consequently a crisis arises unless measures are taken to replete the overtaxed and undernourished tissues by a richer diet.

Further evidence in regard to body stores of calcium has been given in the studies of Sherman and Booher ('31) showing the parallel effect of the level of calcium intake on the bodies of rats. These workers have shown that from external appearances the rats may be apparently well nourished but in reality their bodies may be seriously calcium-poor.

Although there was no measurable maternal growth in stature as shown by height and x-ray of the carpal centers[4] in the subject of this investigation, the large maternal storage seems to point to a filling in of bodily stores, in addition to satisfying fetal requirements when the intake had been raised

[4] The x-ray interpretations for growth were graciously made by Lawrence Reynolds, M.D., roentgenologist of Detroit, and T. Wingate Todd, M.D., of the Brush Foundation of Cleveland.

to a higher level than had previously been maintained. It is possible that as the maternal body itself reached saturation during adequate intake, the retentions would become less high as has been shown by Fairbanks and Mitchell ('36) in rats, by Ellenberger, Newlander and Jones ('32) in cows, by Boldt, Brahm and Andresen ('29) in infants, and by Wang, Kern and Kaucher ('29) in children. The need for knowledge of the nutritional history of the animal with an insight into the state of the body stores was expressed by Hart, Steenbock and Hoppert ('23–'24) when they were using dairy cows in experimental inquiry of maintaining calcium and phosphorus reserves during lactation by the metabolic balance method. Certainly the results recorded herein afford further significant information on women during the reproductive cycle.

Trends in the utilization for the other elements stored by I.M.B. are given in table 1 and show that with a mean daily intake of 0.39, 2.77, 4.05 gm. of magnesium, sodium and potassium, the simultaneous retentions were 0.06, 0.11 and 0.01 gm., respectively. In a like manner, a mean daily intake of 1.93, 3.91 and 1.05 gm. of phosphorus, chlorine and sulfur gave a storage of 0.27, —0.17 and 0.27 gm., respectively. These data are comparable to the other studies in the literature (Hoffström, '10; Coons, Coons and Schiefelbusch, '34; Hummel and co-workers, '36) for magnesium, but lower than those found for the quadripara (L.R.) for sodium and potassium. Furthermore the total daily phosphorus storage was 0.27, 0.30, 0.21 and 0.32 gm. and therefore similar respectively for the cases referred to above. There was an average loss of chlorine by I.M.B. whereas L.R. retained 0.69 gm. daily. Erratic behavior of sodium, potassium and chlorine has been observed by Clark ('26) and Wiley, Wiley and Waller ('33).[6]

The relationship of intake to retention has been the point of interest in most metabolic balance studies for the purpose of establishing fitting dietary standards. There is a wide scatter of retention values in pregnancy on any given level

[6] A detailed report on the metabolism of these elements is forthcoming from the Research Laboratory of the Children's Fund of Michigan.

of intake for both nitrogen and calcium as illustrated in figure 3. This is true not only in isolated balances taken from the literature but also in the present continuous study where the conditions of living and diet were approximately constant.

In summary, the metabolic balance method for determining food standards is not precise and cannot be interpreted too

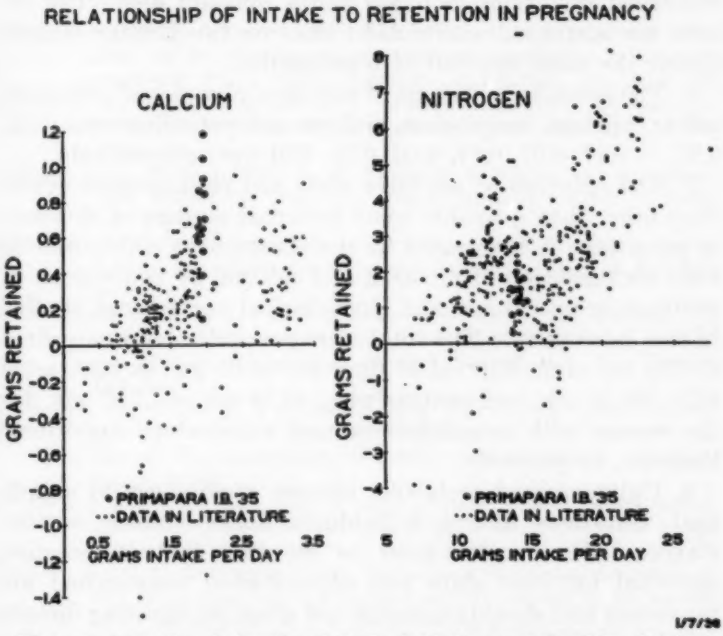

Figure 3 shows the relationship of the level of nitrogen and calcium intake to the amount retained. The results of the present continuous observation in pregnancy are superimposed upon the studies taken from the literature and show not only a wide range of retentions of different individuals on a given level of intake but also significant variations of one individual at different times.

freely without further knowledge of the influencing factors because of such wide physiological variations observed among individuals with highly different nutritive and hereditary backgrounds and of significant fluctuations of the same individual from time to time when maintained under highly controlled environmental conditions and a constant diet.

SUMMARY

1. The present report records the metabolic balances of an 18-year-old primapara observed uninterruptedly during the final 65 days of gestation. Her medical history showed an unsatisfactory nutritional background for 6 preceding years. During the metabolic study she was maintained under highly controlled conditions of living and a constant diet. The results are compared with similar ones on two healthy women during the same interval of reproduction.

2. The mean daily storage of nitrogen, phosphorus, chlorine, sulfur, calcium, magnesium, sodium and potassium was 1.32, 0.27, —0.17, 0.27, 0.71, 0.06, 0.11, 0.01 gm., respectively.

3. The influence of nutritive state and physiological conditions other than gravidity upon maternal storage of elements in pregnancy is illustrated by the conspicuous differences in total accumulation of nitrogen and calcium by two women of contrasting nutritional and physiological background studied in this laboratory. The total nitrogen and calcium acquired during the same interval of time was 86.05 gm. as contrasted with 196.25 gm. and contrariwise, 46.29 gm. vs. 24.7 gm. for the women with unsatisfactory and satisfactory nutritional histories, respectively.

4. Uninterrupted metabolic balance studies reveal significant variations among individuals under similar circumstances. The results point to the fact that in practice, maternal nutritive state and physiological constitution are important and should be considered when interpreting dietary requirements for pregnancy.

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THE DETERMINATION OF VITAMINS B AND G IN HUMAN URINE BY THE RAT-GROWTH METHOD [1]

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ONE FIGURE

(Received for publication September 25, 1936)

Recently interest has been renewed in the quantitative determinations of the B vitamins in human urine. Helmer ('35) reported the presence of vitamins B and G in human urine by the rat-growth method. Harris and Leong ('36) have made extensive studies on the content of vitamin B in human urine in relation to intake. Roscoe ('36) has also presented data on the vitamin B(B_1) and G(B_2) content of human urine, and Emmerie ('36) has determined the flavin content of the urine. The earlier literature has been reviewed by Harris and Leong, and Roscoe.

In our preliminary experiments (Helmer, '35) the whole 24-hour urine sample was concentrated to a thick syrup by vacuum distillation at a low temperature and dried in vacuo over sulfuric acid. The dried and powdered urine was mixed with one part by weight of sucrose and one-half part by weight of hydrogenated vegetable oil[2] and fed in quantities equivalent to one-twenty-fifth of the daily 24-hour urine output to rats on a basal diet. These experiments were not wholly satisfactory because some of the animals would not eat all of the urine supplement. Furthermore, the concentration of the urine by vacuum distillation at a low temperature was a time-consuming procedure. In this communication we wish to report a simplified method of preparing the urine supplements

[1] A report of this work was presented before the American Society of Biological Chemists at Washington, D. C., March, 1936. J. Biol. Chem., vol. 114, p. 48.

[2] Crisco.

by means of adsorption of the vitamins B and G. Harris and Leong ('36) have used a similar technic.

EXPERIMENTAL[1]

The 24-hour urine specimens were adjusted to pH 4.0 by the addition of concentrated hydrochloric acid and then 5 gm. of Lloyd's reagent were added. The urine was stirred for from 15 to 30 minutes with a mechanical stirrer or was allowed to stand in the ice box for 3 or 4 days with occasional shaking. The Lloyd's reagent adsorbate was collected by means of vacuum filtration on hardened filter paper, washed with water, and dried in vacuo over sulphuric acid. As shown in table 2, the 5 gm. of Lloyd's reagent was sufficient to adsorb all of the vitamins B and G from the 24-hour urine sample, inasmuch as a second adsorption with Lloyd's reagent failed to recover any additional quantity of these vitamins.

In the light of the multiple nature of the vitamin G(B_2) complex—György ('35), Birch, György and Harris ('35), Elvehjem and Koehn ('35), Block and Hubbell ('35), Hogan and Richardson ('35), Lepkovsky, Jukes and Krause ('36), and others—the nature of the vitamin supplements for the tests for vitamin B and G had to be considered. The vitamin supplement for the tests for B offered no difficulties since autoclaved yeast should supply the whole vitamin G complex. In the tests for vitamin G in the adsorbates, a supplement was required which would supply the other components of the vitamin G complex necessary for the growth of the rats. In the experiments using the whole dried urine (Helmer, '35), a rice polish extract prepared according to Rosedale ('27) was used for the source of vitamin B. This preparation was a fortunate choice, as shown in figure 1 (group 2), for, when a

[1] In this paper vitamin B refers to the antineuritic vitamin, vitamin G to lactoflavin. The term 'rat factor' may be considered equivalent to 'B_1' or 'rat antiacrodynia factor' of György, Birch, György and Harris, or to the anti-dermatitis factor of Hogan and Richardson, or to the 'third factor' of Block and Hubbell. The term 'chick factor' will refer to the chick anti-pellagra factor B_3 of Elvehjem, P–P (pellagra-preventative) factor, or to the filtrate factor of Lepkovsky and Jukes. By 'the vitamin G complex' is meant the whole of the heat stable fraction of the water-soluble vitamin B complex.

pure vitamin B[4] preparation made by the method of Stuart, Block and Cowgill ('34) was used as a source of vitamin B, a poor growth response was obtained. The substitution of the Rosedale preparation resulted in an increase in growth rate. This rice polish extract evidently contained enough of the 'rat factor' to cause resumption of growth. Birch, György and Harris have shown that rice polishings are a rich source of this factor. It is evidently not precipitated by the lead

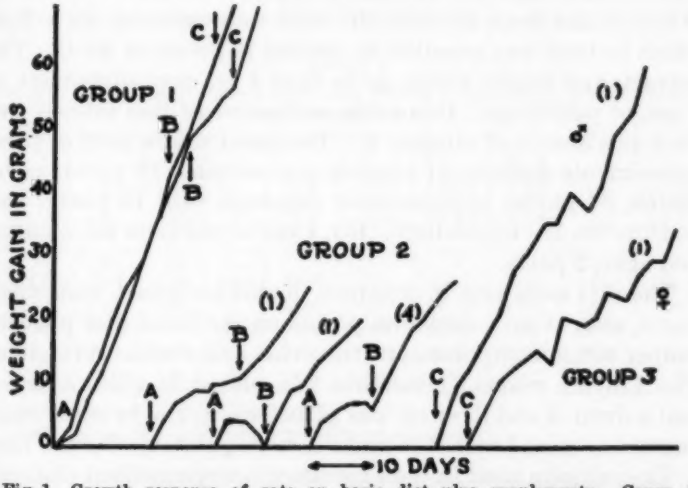

Fig. 1 Growth response of rats on basic diet plus supplements. Group 1, autoclaved yeast plus vitamin B; group 2, Lloyd's reagent adsorbate equivalent to one-twenty-fifth of daily urine output plus vitamin B; group 3, crystalline lactoflavin (0.1 mg.) plus Rosedale's rice polish extract. At A, 1 cc. of a parenteral vitamin B concentrate (diluted 1: 7½); at B, 0.5 cc. of Rosedale's rice polish extract; at C, 1 cc. of Rosedale's rice polish extract.

acetate used in the preparation of this extract. Further evidence is given in figure 1 (group 3) that the rice polish extract of Rosedale's contains the 'rat factor.' Good growth resulted when the basic diet was supplemented with only the Rosedale rice polish extract and crystalline lactoflavin. Figure 1 (group 1) demonstrates that this extract in quantities equivalent to

[4] The author wishes to thank Elmer H. Stuart of Eli Lilly and Company for this preparation.

1 gm. of original rice polishings supplies an adequate amount of vitamin B.

The rice polishing extract was prepared as follows: 500 gm. of rice polishings were extracted with 1% acetic acid by stirring with a mechanical stirrer for 8 hours, the flask being kept at 40°C. After filtering, the extract was concentrated in vacuo at 40°C. to such a volume that 1 cc. was equivalent to 1 gm. of original polishings. Then the extract was treated with lead acetate until no further precipitate could be obtained. When it had been filtered, the lead was removed with H_2S, which in turn was expelled by boiling in vacuo at 40°C. The extract was finally made up so that 1 cc. was equivalent to 1 gm. of polishings. One cubic centimeter of this extract was used as a source of vitamin B. The basal ration used in these experiments contained: vitamin-free casein,[5] 18 parts; cornstarch, 64 parts; hydrogenated vegetable oil,[6] 10 parts; salt mixture no. 185 (McCollum, '18), 4 parts; cod liver oil, 2 parts; and agar, 2 parts.

The rats were kept in separate, double-bottomed, wide mesh cages, and, after a depletion period on the basal diet plus the proper vitamin supplements, the urine adsorbates were given. The Lloyd's reagent adsorbate was placed in glass coasters and a drop of cod liver oil was placed on it, and in some cases sugar was mixed with it to make it more palatable for the rats.

The subjects used in the experiments were medical students and laboratory workers in good health. They were placed on a fixed diet—i.e., one in which the meals were identical in quantity and components three times a day—for a week (see table 1). The urines were collected daily and each 24-hour specimen was treated with Lloyd's reagent, as described above, as soon as each sample was complete. The vitamin B requirement of the subject was calculated by Cowgill's formula ('34). The milligram equivalents (mg.-eq.) excreted in the urine were roughly calculated from the growth data presented in table 2 by first converting to Sherman-Chase units, and then to milligram-equivalents on the basis that one Sherman unit is equivalent to 10 mg.-eq.

[5] The Casein Manufacturing Company of America, Inc., New York.
[6] Crisco.

EXCRETION OF VITAMIN B AND G IN URINE

The growth responses to the urine preparations are shown in table 2, and the relation of the vitamin B excreted to the intake of this vitamin is shown in table 1. The pooled urines of subjects I and II, and the urine of III and of IV contained demonstrable amounts of vitamin B. No significant growth response could be obtained from the urine from subject V when the urine adsorbate was tested for vitamin B. The absence of vitamin B might be due to the fact that so much of the vitamin, calculated by Cowgill's data, was made up of

TABLE 1

The daily caloric and vitamin B intake and vitamin B excretion of normal subjects on a weighed diet

SUBJECT	WEIGHT	HEIGHT	AGE	CALORIC INTAKE	CALCULATED MG.-EQ.[1] INTAKE	CALCULATED MG.-EQ.[1] REQUIREMENT	CALCULATED MG.-EQ.[1] IN EXCESS	MG.-EQ.[1] EXCRETED	PER CENT OF MG.-EQ.[1] INTAKE EXCRETED
	kilos	*cm.*							
I and II	72.6	180	24	2600	7066	5366	1700	1465	21
III	77.2	168	36	2880	7450	6314	1136	1133	15
IVa	76.3	191	19	2600	7022	5706	1360	474	7
IVb	79.5	191	20	3270	9055	7383	1672	1035	10
V	75.0	191	19	3276	8165[2]	6978	1187	±	0

[1] Milligram-equivalents (Cowgill, '34).

[2] 1680 mg.-eq. of total intake were made up of cream.

cream. It is difficult to draw conclusions from these data as to the relation of the amount of vitamin B excreted in the urine to the excess of vitamin B in the diet. However, in three instances the amount of vitamin B in the urine accounts for a considerable proportion of the excess.

The multiple nature of the vitamin G complex makes it difficult at present to make balance studies on vitamin G, since our foodstuffs will have to be reassayed in the light of newer knowledge. The urines from the normal subjects tested so far for vitamins B and G have had in every instance a higher content of vitamin G than of vitamin B.

The urine from subjects I and II, when fed alone as a source of both vitamins B and G, produced a growth response of 6.4

gm. per week. The Lloyd's reagent must have adsorbed some 'rat factor' as well as vitamins B and G. Block and Hubbel ('35) and Lepkovsky et al. ('36) have shown that the 'rat factor' can be adsorbed on fuller's earth. The limiting factor

TABLE 2

The daily excretion of vitamins B and G in the urine of normal subjects

EXPERIMENTAL ANIMAL	SUPPLEMENT	NUMBER OF RATS	DAYS FED	AVERAGE TOTAL GAIN IN WEIGHT	AVERAGE WEEKLY GAIN IN WEIGHT	URINARY VITAMIN B OR G IN SHERMAN UNITS
				gm.	gm.	
+ control	Brewer's yeast	2	35	76	15.0	
+ control	B and autoclaved yeast	3	36	71	12.5	
— control	B	5	42	—1	0.0	
— control	Autoclaved yeast	9	41	4	0.7	
Urine (alone), subject I and II	Urine (1/50)	4	26	24	6.4	
Urine B, subject I and II	Urine (1/50) + autoclaved yeast	5	28	35	8.8	147
Urine G, subject I and II	Urine (1/50 + B	5	28	42	10.5	175
Urine B, subject I and II	Urine (1/25) + autoclaved yeast	3	15	31	13.4	112
Urine G, subject I and II	Urine (1/25) + B	3	15	34	14.4	120
Readsorbed urine B, subject I and II	Urine (1/25) + autoclaved yeast	2	19	—13	—4.8	0
Readsorbed urine G, subject I and II	Urine (1/25) + B	2	20	—4	—1.3	0
Urine B, subject III	Urine (1/50) + autoclaved yeast	2	28	27	6.8	113
Urine B, subject IVa	Urine (1/33) + autoclaved yeast	2	29	18	4.4	47
Urine B, subject IVb	Urine (1/50) + autoclaved yeast	2	28	24	6.2	104
Urine G, subject Vb	Urine (1/50) + autoclaved yeast	2	28	45	11.1	185
Urine G, subject Vb	Urine (1/75) + autoclaved yeast	3	28	28	7.0	175

in the growth response by the urine adsorbates alone may be the 'rat factor' and 'chick factor.' More observations will have to be made with purified supplements to determine this point.

DISCUSSION

The results in the experiments reported in this paper agree with those of Harris and Leong ('36) using 'acid clay' for the adsorption of vitamin B and the 'bradycardia' method of Harris for determining the vitamin B. His method has the advantage over the 'rat-growth' method of requiring less urine for the test. The concentration of the vitamins B and G by means of adsorption methods has the advantage over the vacuum distillation methods in that larger amounts of urine can be given without the toxic effects that Roscoe ('36) described in her experiments with the urine concentrated by vacuum distillation. For example, better growth was obtained in the experiments reported in this paper with one-fiftieth of the daily urinary output than was obtained in those with one-twenty-fifth of the daily output using the urine concentrated by vacuum distillation (Helmer, '35).

Harris and Leong ('36) reported that 5 to 8% of the vitamin B ingested was excreted in the urine. Considering the differences in method, most of the figures reported here are not in great variance with those of the above authors. In subjects I and II, III and IVb the vitamin B excreted in the urine agrees quite well with the calculated excess of the vitamin. A greater discrepancy occurred with subject IV with diet IVa. He is a very active individual, and the diet of 2600 calories did not satisfy his appetite. The additional caloric intake of diet Vb satisfied his appetite, and, although the calculated milligram-equivalent excess of vitamin B was not greatly increased, considerably more of the excess was excreted in the urine.

The results in this paper also agree with those reported earlier by Helmer ('35) and with those of Roscoe ('36) and Emmerie ('36) that vitamin G or flavin is excreted in normal urine.

SUMMARY

The vitamins B and G of normal human urine have been adsorbed on Lloyd's reagent and tested for activity by the rat-growth method. When an excess of vitamin B, calculated by Cowgill's formula, was present in the diet, vitamin B could be detected in definite quantities in the urine. Vitamin G was present in the normal urine tested in larger amounts than vitamin B.

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THE INFLUENCE OF CARBOHYDRATE ON NITROGEN METABOLISM IN THE NORMAL NUTRITIONAL STATE

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It is now well known that in certain abnormal nutritional states the amount of carbohydrate ingested conditions the extent of protein catabolism. Thus, during fasting and specific nitrogen hunger, carbohydrate prevents the breakdown of body protein (Lusk, '28 a; Landergren, '03; Murlin, '07; Cathcart, '09). More striking, however, is the relation of carbohydrate to the excessive protein metabolism of the phlorhizinized dog. In such an animal, glucose spares protein despite the fact that the glucose producing this effect is being excreted (Ringer, 12; Deuel and Chambers, '25; Gaebler and Murlin, '25). Although the mechanism whereby carbohydrate spares protein is still obscure, this experiment leaves little doubt that it is not always due to an isodynamic replacement of the carbohydrate for the protein that fails to be metabolized. The value of carbohydrate as an inhibitor of still another form of protein destruction, namely, that found in febrile conditions and in hyperthyroidism, has been demonstrated. In both conditions nitrogen equilibrium can be obtained by the use of high calorie diets containing large amounts of carbohydrates (Shaffer, '08; Shaffer and Coleman, '09; Kendall, '29).

The influence of carbohydrate in apparently normal nutritional states was recognized as early as 1856 by Hoppe who showed that carbohydrate lowered the nitrogen excretion in

dogs maintained on an exclusively meat diet. This observation was later confirmed by Lusk (1890) among others.

In the present investigation normal dogs were brought into nitrogen equilibrium by being fed the synthetic mixture devised by Cowgill ('23). By this procedure an equilibrium was established in which the 24-hour nitrogen excretion over long periods of time rarely varied by more than 0.30 gm. The maintenance of this degree of constancy provided an experimental animal for the detection of unusually small changes in nitrogen elimination. At various intervals following the ingestion of the meal, additional quantities of carbohydrate were fed. It was found that the occurrence as well as the degree of nitrogen storage effected by single feedings of carbohydrate was related to the time that elapsed between the consumption of the daily meal and the ingestion of the extra carbohydrate. In the normal nutritional state, carbohydrate does not spare protein unless fed at certain intervals relative to the ingestion of protein. The present study thus establishes a new factor, namely one of time, in the protein-sparing action of carbohydrate under the conditions of nitrogen equilibrium.

EXPERIMENTAL

Care of animals. Adult female dogs were used. They were kept in metal metabolism cages and fed once per day a synthetic diet devised by Cowgill ('23). The casein, sucrose, vitavose, bone ash and Karr's salt mixture ('20) were thoroughly mixed in quantities sufficient for several complete experiments. The uniformity of the mixture was tested by nitrogen determinations in various parts. Fat consisting of 17 gm. of lard and 9 gm. of unsalted butter per 100 gm. of the above mixture was added at each time of feeding. Each dog, receiving about 60 calories per kilogram of body weight, maintained a good appetite throughout the experiment, ingesting within a few minutes all food served. By this procedure, the animals were brought into nitrogen equilibrium after 10 days or 2 weeks of feeding; after this period the

24-hour excretion of nitrogen rarely varied by more than 0.30 gm. of nitrogen. Four days or more of an equilibrium in which the daily excretion of nitrogen did not vary by more than 0.20 gm. were accepted as a suitable control period prior to the feeding of the extra carbohydrate. No nitrogen observations were made near to or during the period of estrus.

The dogs were fed once per day, always in the morning, and catheterized just before the meal was served. In the animals that were subjected to catheterization twice daily, the second was carried out in the evening, exactly 12 hours after the first. Care was taken to maintain environmental conditions (such as temperature of the room, times of feedings and catheterizations) as constant as possible throughout each period of observation.

Methods. The following analytical procedures for urine were employed: ammonia, Folin and Bell method ('17); urea, Van Slyke method ('32); total nitrogen, Kjeldahl method; inorganic sulfur, Rosenheim and Drummond method ('14).

Feces were marked by means of carmine and stored in concentrated sulfuric acid. Its nitrogen content was determined by a modification of the alternative procedure described by Peters and Van Slyke ('32).

I. The effect produced on the 24-hour nitrogen excretion by a single ingestion of 50 extra gm. of glucose with the meal and at various intervals before and after the meal

The three dogs recorded in tables 1, 2 and 3 were catheterized once daily and shortly thereafter (15 to 25 minutes) fed the regular daily meal. The term '24-hour period' refers to the interval between two catheterizations, and it should be noted that the daily meal was ingested at the beginning of each 24-hour period. Since in each dog the time of the ingestion of the daily meal was kept constant throughout each period of observation, the time of the administration of the 50 extra gm. of carbohydrate has been expressed as time before the next or after the last meal. Extra glucose was administered at the following intervals: 1) With the meal.

2) At 1, 2, 3, 4 and 10 hours after the last meal. 3) At 1, 2, 4 and 6 hours before the next meal (i.e., 23, 22, 20 and 18 hours, respectively, after the last meal).

Each change in nitrogen excretion—when it occurred in response to the carbohydrate—lasted in most cases for 48 hours and consisted of two distinct phases: 1) A drop below the previously established equilibrium level; this was observed in the first 24-hour period. 2) A rise to a level above that of the resting equilibrium; this was found in the following 24-hour period.

With the meal. The response of urinary nitrogen to the administration of 50 gm. of glucose along with the daily meal was tested in three dogs. In dog O (table 1) this led to a drop from a resting equilibrium level of 5.21–5.37 to 4.70 gm. in the 24-hour period in which the carbohydrate was ingested. In the following 24-hour period total nitrogen rose to 5.67 gm. In the third 24-hour period the nitrogen excretion had returned to the resting level. These changes in total nitrogen generally reflect changes in urea excretion. Dogs B (table 2) and T (table 3) showed similar responses.

One hour after the last meal. A single observation on the administration of extra carbohydrate at this interval was made in dog O. There was again observed the decrease in nitrogen excretion in the 24-hour period in which the carbohydrate was ingested, and this was followed in the next 24 hours by the compensatory rise to a level in excess of the previously established equilibrium.

Two hours after the meal. While both phases of the action of carbohydrate on nitrogen excretion were found in dogs B and O when the extra glucose was fed at this interval, the extent of the initial (first 24-hour period) depression in nitrogen output was not so great as that found when carbohydrate was fed either with or at earlier intervals after the meal. Thus, drops to the extent of 0.50 (dog B) and 0.60 gm. (dog O) followed the ingestion of 50 extra gm. of glucose with the meal, whereas the ingestion of the same amount of carbohydrate 2 hours later led to decreases in nitrogen excretion to the extent of 0.20 and 0.35 gm., respectively.

Three hours after the meal. Both the initial fall and compensatory rise in nitrogen excretion were again observed when carbohydrate was fed at this interval (table 1), but the effects were not so marked as those observed when carbohydrate was fed at the first two intervals recorded above.

TABLE 1

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog O

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	*gm.*	*gm.*
1		4.30	0.554	5.33
2		4.37	0.542	5.37
3		4.28	0.528	5.24
4		4.25	0.571	5.28
5	10 hours after meal	4.33	0.516	5.26
6		4.22	0.539	5.21
7		4.36	0.547	5.34
8	With meal	3.88	0.501	4.70
9		4.65	0.551	5.67
10		4.34	0.531	5.31
11		4.28	0.584	5.32
12		4.35	0.521	5.35
13	1 hour after meal	3.77	0.561	4.78
14		4.67	0.552	5.69
15		4.54	0.507	5.50
16		4.32	0.555	5.30
17	2 hours after meal	3.98	0.488	4.95
18		4.73	0.541	5.73
19		4.27	0.573	5.27
20		4.40	0.533	5.35
21		4.43	0.556	5.40
22	3 hours after meal	4.16	0.509	5.11
23		4.62	0.527	5.63
24		4.51	0.542	5.48
25		4.38	0.518	5.39
26	4 hours after meal	4.23	0.526	5.23
27		4.30	0.502	5.25
28		4.35	0.534	5.36

Catheterized at 7.45 A.M. daily; fed at 8.00 A.M. daily.
Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter.
Diet nitrogen = 5.54 gm./100 gm. of diet mixture.
This dog maintained a constant weight of 9.6 kg. throughout the experiment.

Four hours after the last meal. Dog O failed to show a definitely measurable response in the nitrogen excretion to the ingestion of 50 gm. of glucose at this time. Although a slight drop may have occurred in dog B, it should be noted

TABLE 2

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog B

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	gm.	gm.
1		5.45	0.505	6.53
2		5.40	0.500	6.47
3		5.34	0.493	6.42
4		5.32	0.477	6.40
5	4 hours after meal	5.18	0.438	6.19
6		5.38	0.525	6.48
7		5.26	0.527	6.39
8	With meal	4.86	0.510	5.88
9		5.88	0.502	6.98
10		5.30	0.534	6.40
11		5.33	0.498	6.39
12	10 hours after meal	5.41	0.537	6.56
13		5.26	0.501	6.34
14		5.32	0.492	6.37
15	2 hours after meal	5.11	0.514	6.16
16		5.59	0.531	6.70
17		5.30	0.522	6.39
18		5.41	0.540	6.53
19		5.40	0.497	6.46
20	4 hours before meal[1]	4.84	0.504	5.90
21		5.35	0.489	6.43
22		5.39	0.553	6.54

Catheterized at 8.35 A.M. daily; fed at 9.00 A.M. daily.
Diet: 125 gm. of diet mixture plus 22 gm. of lard plus 11 gm. of unsalted butter.
Diet nitrogen = 5.66 gm./100 gm. of diet mixture.
This dog maintained a constant weight of 11.5 kg. throughout the experiment.
[1] Twenty hours after the meal fed on day 19.

that a measureable compensatory rise was not observed in the following 24 hours.

Ten hours after the last meal. Dogs B, O and T showed no effect whatsoever of the ingestion of the extra carbohydrate at this interval. Thus in dog O, the equilibrium level was

TABLE 3

The effect of a single ingestion of 50 gm. of glucose upon the 24-hour nitrogen excretion of dog T

DAY	TIME CARBOHYDRATE FED	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN
		gm.	*gm.*	*gm.*
1		4.56	0.392	5.43
2		4.53	0.402	5.45
3		4.41	0.388	5.30
4		4.47	0.420	5.36
5		4.80	0.407	5.71
6		4.43	0.452	5.34
7	10 hours after meal	4.59	0.397	5.44
8		4.45	0.421	5.36
9		4.40	0.436	5.34
10		4.48	0.413	5.38
11	With meal	3.94	0.417	4.82
12		5.01	0.392	5.93
13		4.62	0.403	5.49
14		4.51	0.416	5.38
15		4.60	0.431	5.52
16		4.56	0.417	5.42
17	1 hour before meal[1]	4.14	0.436	5.06
18		4.90	0.432	5.85
19		4.72	0.418	5.60
20		4.58	0.401	5.48
21		4.52	0.436	5.49
22	2 hours before meal[2]	4.12	0.413	5.00
23		4.58	0.402	5.45
24		4.48	0.413	5.36
25		4.58	0.422	5.50
26	4 hours before meal[3]	4.24	0.435	5.14
27		4.82	0.424	5.66
28		4.61	0.403	5.49
29		4.49	0.430	5.41
30		4.60	0.422	5.47
31		4.47	0.416	5.37
32	6 hours before meal[4]	4.40	0.428	5.36
33		4.58	0.419	5.46

Catheterized at 7.45 A.M. daily; fed at 8.00 A.M. daily.

Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter.

Diet nitrogen = 5.45 gm./100 gm. of diet mixture.

Weight of this dog on first and last day of experiment was 10.2 kg. and 10.3 kg., respectively.

[1] Twenty-three hours after the meal fed on day 16.

[2] Twenty-two hours after the meal fed on day 21.

[3] Twenty hours after the meal fed on day 25.

[4] Eighteen hours after the meal fed on day 31.

5.24–5.37 gm. before glucose administration, while on the day during and the day following the ingestion of carbohydrate the 24-hour excretions of nitrogen were respectively 5.26 and 5.21 gm.

Eighteen hours after the last meal or 6 hours before the next meal. As in the case of the 10-hour interval, carbohydrate had no effect on nitrogen excretion when its ingestion was delayed for 18 hours after the meal (table 3).

Twenty hours after the last meal or 4 hours before the next meal. Although carbohydrate fed at this interval (dogs B and T) influenced the nitrogen excretion, an important difference was observed between the nitrogen spared by early and late feeding of extra carbohydrate. This difference consisted in the time of onset of the change. When the extra carbohydrate was fed 1 to 4 hours after the meal, the initial effect, namely the drop in nitrogen excretion, occurred during the same 24-hour period in which the extra carbohydrate was administered. But when ingested 20 hours after the last meal (i.e., 4 hours before the next meal) the 50 extra gm. of glucose failed to influence the nitrogen excreted during the same 24-hour period in which the extra carbohydrate was ingested, the initial drop occurring in the nitrogen of the following 24 hours. It was the nitrogen to which the next meal contributed that was affected by this late feeding of extra carbohydrate.

This delayed sparing of nitrogen by carbohydrate is more strikingly brought out in the following experiments, in which the time at which the extra carbohydrate was fed more closely approached the ingestion of the next regular meal. These results demonstrate that carbohydrate spares the nitrogen of dogs brought into nitrogen equilibrium only when fed at certain intervals related to the time of the ingestion of the daily meal.

Two hours before the next meal or 22 hours after the last meal. A delayed but nevertheless definite nitrogen response was obtained when extra carbohydrate was fed at this interval (table 3).

One hour before the next meal or 23 hours after the last meal. When fed approximately ¾ hour before the next catheterization, carbohydrate again failed to influence the nitrogen excreted in the 24-hour period in which the extra carbohydrate was ingested (dog T). During the following 24 hours, however, the total nitrogen fell from an equilibrium level of 5.38–5.52 to 5.06 gm.

II. The effect produced by a single feeding of 50 gm. extra glucose at various intervals upon the first and second 12-hour excretions of nitrogen

Since a compensatory rise in the excretion of nitrogen followed in many cases the sparing effect of carbohydrate, it was conceivable that the failure to find a retention of nitrogen in the 24-hour urine sample when carbohydrate was administered at later intervals may have been due to a balance between a decreased and an increased nitrogen output. That this is not the case, however, is shown in the experiments recorded in table 4, in which nitrogen was studied over shorter intervals after carbohydrate administration. Each dog was catheterized at exactly 12-hour intervals. The term 'first 12-hour period' refers to the interval between 7.40 A.M. and 7.40 P.M. (dog L), whereas the 'second 12-hour period' refers to the interval from 7.40 P.M. to 7.40 A.M. Extra glucose was fed at two times: 1) at the same time as the meal, and 2) 12 hours after the meal. The extra carbohydrate was therefore administered at the beginning of both periods in which 12-hour urine samples were collected.

1. With the meal. Although, as already noted, 50 gm. of extra glucose administered at the same time as the meal led to a drop in the urea as well as in the total nitrogen measured in the 24-hour urine sample, nevertheless a decreased nitrogen output does not characterize the nitrogen eliminated throughout the whole of the 24-hour period of observation. A marked drop (0.68 gm. in dog L; 0.91 gm. in dog T; 0.87 gm. in dog S) occurred during the 'first 12-hour period,' at the beginning of which the extra carbohydrate was fed. In the next 12-hour

TABLE 4

The effect of a single feeding of 50 gm. of extra glucose at various intervals upon the first and second 12-hour excretion of nitrogen

	DAY	TIME CARBOHYDRATE FED	UREA NITROGEN			AMMONIA NITROGEN			TOTAL NITROGEN		
			First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dog L	1		3.49	1.75	5.24	0.275	0.219	0.494	4.03	2.30	6.33
	2		3.54	1.90	5.44	0.218	0.178	0.396	4.03	2.41	6.44
	3		3.38	1.91	5.29	0.260	0.217	0.477	3.91	2.42	6.33
	4		3.35	1.84	5.19	0.234	0.215	0.449	3.98	2.29	6.27
	5	With meal	2.76	2.17	4.93	0.213	0.219	0.432	3.30	2.65	5.95
	6		3.43	2.13	5.56	0.232	0.176	0.408	3.98	2.54	6.52
	7		3.45	1.90	5.35	0.276	0.222	0.498	4.10	2.38	6.48
	8		3.41	1.92	5.33	0.258	0.189	0.447	4.02	2.41	6.43
	9	12 hours after meal	3.46	1.79	5.25	0.255	0.198	0.453	3.98	2.31	6.29
	10		3.37	1.88	5.25	0.241	0.203	0.444	3.91	2.40	6.31
Dog T	1		3.19	1.61	4.80	0.292	0.178	0.470	3.73	2.06	5.79
	2		3.18	1.55	4.73	0.305	0.134	0.439	3.80	1.90	5.70
	3		3.29	1.59	4.88	0.275	0.162	0.437	3.78	1.96	5.74
	4		3.30	1.53	4.83	0.298	0.152	0.450	3.85	1.93	5.78
	5	With meal	2.44	1.80	4.24	0.252	0.167	0.419	2.94	2.21	5.15
	6		3.21	1.87	5.08	0.234	0.167	0.401	3.75	2.23	5.98
	7		3.57	1.54	5.11	0.334	0.125	0.459	4.22	1.82	6.04
	8		3.28	1.60	4.88	0.289	0.155	0.444	3.87	1.96	5.83
	9		3.32	1.55	4.87	0.294	0.153	0.447	3.88	1.91	5.79
	10	12 hours after meal	3.23	1.65	4.88	0.256	0.178	0.434	3.80	2.05	5.85
	11		3.19	1.62	4.81	0.266	0.125	0.391	3.75	1.97	5.72
Dog S	1		2.03	1.34	3.37	0.173	0.173	0.346	2.43	1.71	4.14
	2		2.02	1.35	3.37	0.173	0.176	0.349	2.44	1.69	4.13
	3		2.08	1.25	3.33	0.166	0.175	0.341	2.45	1.61	4.06
	4		2.15	1.28	3.43	0.172	0.177	0.349	2.54	1.66	4.20
	5	With meal	1.32	1.63	2.95	0.144	0.169	0.313	1.67	1.99	3.66
	6		2.17	1.61	3.78	0.165	0.179	0.344	2.55	2.02	4.57
	7		2.08	1.29	3.37	0.162	0.172	0.334	2.48	1.65	4.13

Dog L—This dog was catheterized at 7.40 A.M. and 7.40 P.M. daily; fed at 8.00 A.M. daily. Diet: 125 gm. of diet mixture plus 23 gm. of lard plus 12 gm. of unsalted butter. Diet nitrogen = 5.44 gm./100 gm. of diet mixture. This dog maintained a constant weight of 12.2 kg. throughout the experiment.

Dog T—This dog was catheterized at 7.45 A.M. and 7.45 P.M. daily; fed at 8.00 A.M. daily. Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter. Diet nitrogen = 5.62 gm./100 gm. of diet mixture. This dog maintained a constant weight of 10.3 kg. throughout the experiment.

Dog S—This dog was catheterized at 9.00 A.M. and 9.00 P.M. daily; fed at 9.15 A.M. daily. Diet: 90 gm. of diet mixture plus 16 gm. of lard plus 8 gm. of unsalted butter. Diet nitrogen = 5.64 gm./100 gm. of diet mixture. This dog maintained a constant weight of 9.2 kg. throughout the experiment.

period, however, the nitrogen excreted rose above that excreted in the 'second 12-hour period' of the previous day. Following the retention, the nitrogen excretion of the 'first 12-hour period' returned to the equilibrium level the next day; the nitrogen output of the 'second 12-hour period' remained elevated in all three animals for 2 days as a result of the extra carbohydrate received. The compensatory rise in nitrogen excretion began early after nitrogen retention, and this indicates the inability of the organism to effect permanent storage of spared protein under the conditions of this study.

2. Twelve hours after the meal. No measurable change was observed in the nitrogen metabolism of the 'first' or 'second 12-hour period' when the extra carbohydrate was ingested at the beginning of the 'second 12-hour period.'

III. The effect of continued feeding of extra carbohydrate on nitrogen excretion

1. Twenty-four hour nitrogen excretion. In the preceding sections single feedings of extra carbohydrate were dealt with. In the following experiments (tables 5, 6 and 7) the effects of continued daily additions of extra carbohydrate along with the regular diet were studied. Four dogs were employed, of which dogs T, E and S received the Cowgill diet in the manner described in the early part of this paper; dog Y (table 7) received a high protein diet (over 60% in the form of protein), which was fed at 12-hour intervals.

In all four dogs the ingestion of extra carbohydrate at the same time as the regular meal resulted in a decreased nitrogen elimination in the 24-hour urine sample, and this decreased level was maintained throughout the whole period in which the extra carbohydrate was ingested. The most striking fall in total nitrogen as well as in urea occurred in dog Y, in which the 24-hour nitrogen excretion fell from a resting level of 11.43–11.60 to 10.26 gm. on the second day of carbohydrate administration.

2. First and second 12-hour nitrogen excretion. Despite the fact that the 24-hour urine sample showed a nitrogen retention throughout the 4 to 7 days in which the extra carbohydrate was ingested, a lowered nitrogen level did not

TABLE 5

Effect of continued feeding of extra glucose upon nitrogen excretion of dog T

DAY	UREA NITROGEN			AMMONIA NITROGEN			TOTAL NITROGEN			EXTRA GLUCOSE [1]	FECAL NITROGEN	WEIGHT
	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kg.
1	3.26	1.79	5.05	0.304	0.199	0.503	3.83	2.18	6.01	..	Total for	12.2
2	3.18	1.82	5.00	0.286	0.195	0.481	3.76	2.22	5.98	..	first 4	...
3	3.22	1.73	4.95	0.303	0.208	0.511	3.78	2.14	5.92	..	days =	12.2
4	3.20	1.71	4.91	0.278	0.200	0.478	3.76	2.10	5.86	..	1.57	...
5	2.24	2.03	4.27	0.208	0.205	0.413	2.67	2.45	5.12	50	Total for	12.3
6	2.03	2.18	4.21	0.189	0.208	0.397	2.42	2.62	5.04	50	second 4	...
7	2.16	2.12	4.28	0.196	0.204	0.400	2.59	2.51	5.10	50	days =	12.3
8	2.36	2.04	4.40	0.215	0.208	0.423	2.89	2.47	5.36	50	1.59	...
9	2.37	1.95	4.32	0.218	0.203	0.421	2.83	2.37	5.20	50		12.4
10	2.39	2.15	4.54	0.212	0.207	0.419	2.86	2.56	5.42	50		...
11	2.48	2.02	4.50	0.200	0.214	0.414	2.90	2.44	5.34	50		12.5
12										50		
13										50		
14										50		
15	3.71	1.83	5.54	0.284	0.169	0.453	4.24	2.23	6.47	..		12.6
16	3.80	1.76	5.56	0.296	0.202	0.498	4.37	2.18	6.55	..		...
17	3.81	1.71	5.52	0.327	0.191	0.518	4.37	2.12	6.49	..		12.6
18	3.52	1.75	5.27	0.321	0.204	0.525	4.08	2.15	6.23	..		...
19	3.22	1.79	5.01	0.319	0.194	0.513	3.76	2.17	5.93	..	Total for	12.6
20	3.31	1.79	5.10	0.331	0.197	0.528	3.89	2.21	6.10	..	4 days =	...
21	3.19	1.85	5.04	0.317	0.203	0.520	3.74	2.24	5.98	..	1.68	12.6

Catheterized daily at 9.00 A.M. and 9.00 P.M. Fed at 9.15 A.M. daily.
Diet: 110 gm. of diet mixture plus 20 gm. of lard plus 10 gm. of unsalted butter.
Diet nitrogen = 5.77 gm./100 gm. of diet mixture.

[1] Fed with the daily meal.

characterize the nitrogen excretion at all intervals of the 24 hours. In dogs T, E and S the nitrogen output was observed at 12-hour intervals. Although the total nitrogen for the 24-hour period showed a significant drop on all days in which

30 or 50 additional grams of glucose were administered, no measurable decrease was observed during the second 12 hours of the day. It was in the first 12 hours that the whole of the

TABLE 6

Effect of prolonged feeding of glucose on urinary and fecal nitrogen excretion of dogs S and E

	DAY	UREA NITROGEN			AMMONIA NITROGEN			TOTAL NITROGEN			EXTRA GLUCOSE [1]	FECAL NITROGEN
		First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours	First 12 hours	Second 12 hours	24 hours		
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dog S	1	1.99	1.52	3.51	0.148	0.154	0.302	2.35	1.89	4.24		Total for
	2	1.94	1.50	3.44	0.144	0.177	0.321	2.32	1.88	4.20		first 4
	3	1.96	1.49	3.45	0.150	0.162	0.312	2.30	1.85	4.15		days =
	4	2.01	1.57	3.58	0.147	0.184	0.331	2.38	1.95	4.33		1.56
	5	1.46	1.59	3.05	0.139	0.175	0.314	1.79	1.96	3.75	30	Total for
	6	1.68	1.51	3.19	0.117	0.178	0.295	1.98	1.89	3.87	30	second 4
	7	1.72	1.50	3.22	0.105	0.184	0.289	2.03	1.90	3.93	30	days =
	8	1.67	1.54	3.21	0.103	0.179	0.282	1.99	1.92	3.91	30	1.66
Dog E	1	3.56	2.25	5.81	0.313	0.183	0.496	4.13	2.71	6.84		Total for
	2	3.58	2.20	5.78	0.313	0.208	0.521	4.17	2.64	6.81		first 4
	3	3.52	2.23	5.75	0.319	0.198	0.517	4.08	2.68	6.76		days =
	4	3.57	2.15	5.72	0.292	0.195	0.487	4.16	2.58	6.74		2.04
	5	3.14	2.20	5.34	0.295	0.198	0.493	3.69	2.64	6.33	30	Total for
	6	3.06	2.40	5.46	0.222	0.214	0.436	3.55	2.86	6.41	30	second 4
	7	3.15	2.04	5.19	0.216	0.199	0.415	3.63	2.52	6.15	30	days =
	8	3.36	2.28	5.64	0.218	0.202	0.420	3.83	2.72	6.55	30	1.80

Dog S—Catheterized at 9.00 A.M. and 9.00 P.M. daily; fed at 9.15 A.M. daily. Diet: 90 gm. of diet mixture plus 16 gm. of lard plus 8 gm. of unsalted butter. Diet nitrogen = 5.77 gm. per 100 gm. of diet mixture. Weight of dog 9.7 kg.

Dog E—Catheterized at 9.05 A.M. and 9.05 P.M. daily; fed at 9.15 A.M. daily. Diet: 130 gm. of diet mixture plus 24 gm. of lard plus 12 gm. of unsalted butter. Diet nitrogen = 5.77 gm. per 100 gm. of diet mixture. Weight of dog 13.0 kg.

[1] Fed with the daily meal.

nitrogen retention occurred. Further evidence that the nitrogen retention under the influence of carbohydrate occurs over a short interval was found in dog T, in which the effects of 50 extra gm. of glucose were followed for 7 days. In this dog, although the nitrogen elimination of the first 12 hours

fell from an equilibrium level of 3.76–3.83 (observed prior to the extra carbohydrate feeding) to values between 2.42 and 2.90 gm., a rise instead of a fall was shown in the nitrogen excretion of the second 12 hours.

TABLE 7

Effect of prolonged feeding of carbohydrate on the nitrogen excretion of dog Y on high protein diet

DAY	UREA NITROGEN	AMMONIA NITROGEN	TOTAL NITROGEN	INORGANIC SULFUR	SUCROSE ADDED
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1	10.00	0.668	11.60	...	
2	9.96	0.810	11.50	...	
3	10.02	0.775	11.44	0.61	
4	10.03	0.785	11.43	0.60	
5	9.44	0.840	11.04	0.56	30[1]
6	8.93	0.752	10.26	0.52	30
7	9.09	0.742	10.51	0.53	30
8	9.42	0.794	10.90	0.54	30
9	9.30	0.794	10.75	0.54	30
10	9.30	0.814	10.75	0.53	30
11	9.57	0.820	11.04	0.56	30
12	10.24	0.870	11.71	0.62	
13	10.64	0.862	12.06	0.63	
14	10.64	0.833	12.07	0.63	
15	10.71	0.901	12.16	0.64	
16	10.58	0.855	12.01	0.63	
17	10.35	0.800	11.73	0.63	
18	9.92	0.787	11.30	0.63	
19	9.80	0.787	11.53	0.61	
20	10.31	0.787	11.82	0.63	
21	10.35	0.782	11.76	0.61	

Weight of dog 7.9 kg.

Diet: casein 59.0%; Cellu-flour 23.5%; cane sugar 11.8%; pancreatin 2.0%; rice bran 2.3%; Karr's salt mixture 1.4%.

Diet nitrogen = 7.77 gm./100 gm. of diet mixture.

Fed 80 gm. of the above mixture at 8.15 A.M. and 8.15 P.M. daily; catheterized at 8.00 A.M. daily.

[1] Fifteen grams sucrose added to diet at each time of feeding.

The cessation of the daily administration of extra carbohydrate led to a most unexpected change in nitrogen excretion. This did not proceed directly from its depressed level to the original equilibrium level established prior to the carbohydrate treatments. In both animals studied (dogs Y and T)

the original nitrogen levels were finally attained after the extra carbohydrate had been withdrawn, but this was preceded by a period in which the nitrogen excreted was definitely in excess of the initial level. For the first 5 days following the removal of the extra carbohydrate, the 24-hour total nitrogen excreted by dog Y (table 7) was markedly above the original equilibrium level (11.43–11.60 gm.), reaching a value of 12.16 on the fourth day. In dog T (table 5) the nitrogen elimination was above the first equilibrium level for 4 days after the extra carbohydrate had been withdrawn. Interestingly enough, this elevation in nitrogen was reflected only in the nitrogen elimination of the first 12 hours, that of the second 12 hours being established at the resting equilibrium level as soon as the extra carbohydrate was removed.

The influence of carbohydrate on the excretion of inorganic sulfur in the urine was determined in dog Y (table 7). Changes in this constituent paralleled those in nitrogen. Thus the administration of the extra sucrose depressed the sulfur excretion from an equilibrium level of 0.61–0.60 to 0.56–0.52 gm. Its excretion remained depressed throughout the whole period in which the extra carbohydrate was ingested, returning to its former level immediately following the interruption of the extra carbohydrate feeding.

In dogs T, E and S, fecal nitrogen was determined at intervals before, during and after the administration of the extra carbohydrate. It should be observed that the extra carbohydrate had no effect on the nitrogen eliminated in the feces.

SUMMARY AND DISCUSSION

From the experimental results presented, some of the limiting factors as well as characteristic features regarding the mechanism whereby carbohydrate spares nitrogen in the normal nutritive state may now be pointed out:

1. The single administration of extra carbohydrate in the normal nutritive state spares nitrogen only when it is ingested at a definite time in relation to the ingestion of protein. When the extra carbohydrate is administered at intervals

far removed from the time of protein feeding, no nitrogen retention occurs even though the amount of extra carbohydrate represents 200 calories or approximately one-third of the daily caloric intake. The interval during which the extra carbohydrate is able to exert a nitrogen-sparing action is limited to 4 hours before and 4 hours after the ingestion of the daily meal. In every case it is the nitrogen to which the protein of the meal contributes that is affected by the extra carbohydrate. This observation thus establishes a new factor, namely, one of time, in the protein-sparing action of carbohydrate in the normal nutritive state. Since, as judged by the nitrogen excretion, the metabolism of protein proceeds most rapidly in the early hours after its ingestion (Lusk, '28 b), this time factor suggests that carbohydrate effectively spares protein only if it is available to the animal at a time when an increase protein metabolism is in progress.

2. Not only the occurrence but also the extent of the nitrogen retention produced by extra carbohydrate administered during the normal nutritive state are determined by the time interval between the ingestion of protein and that of carbohydrate. The most pronounced sparing of nitrogen occurs when the extra carbohydrate is ingested at the same time as the meal or within 1 hour after it. As the interval between the ingestion of these two foodstuffs is lengthened, the nitrogen spared by equal amounts of carbohydrate falls off until finally, when the interval between the two is greater than 4 hours, no sparing whatever occurs.

3. Despite the fact that the 24-hour nitrogen output may show a well-defined sparing of nitrogen in response to a single administration of extra carbohydrate, a decreased nitrogen excretion does not characterize the whole of the 24-hour period during which the carbohydrate is ingested. The interval in which nitrogen retention occurs after a single administration of 50 gm. of extra carbohydrate is very short. In the present study it was observed during the first 12 hours following the carbohydrate feeding. The nitrogen eliminated in the next 12 hours had already shown a rise above the previously established equilibrium level for this period. The nitrogen spared

under the influence of a single feeding of extra carbohydrate is therefore not permanent, its elimination beginning several hours after its storage is effected.

4. Repeated daily additions of extra carbohydrate at the same time that protein is ingested result in nitrogen retention, but this continues only as long as the daily administrations of extra carbohydrate are maintained. The retention of this nitrogen requires the continued presence of the extra carbohydrate, for, although nitrogen continued to be deposited for 7 days under the influence of repeated administrations of extra carbohydrate, the cessation of this extra carbohydrate results in an immediate outpouring of increased amounts of nitrogen, a process that continues for several days after the feeding of extra carbohydrate is discontinued. Here again, despite the fact that the administration of extra carbohydrate was repeated daily for 7 days, actual storage of nitrogen occurred only during the first few hours following the carbohydrate intake, the nitrogen excretion during the later intervals of the 24-hour period being relatively unaffected or even increased. Similarly, when an increased outpouring of nitrogen results from the cessation of daily carbohydrate treatments, this takes place in the interval corresponding to that in which the nitrogen storage is observed (i.e., the first 12 hours), the nitrogen excretion of the later periods remaining relatively unaffected. The fact that an increased nitrogen output in the early hours after protein ingestion was observed for several days after an interruption of extra carbohydrate feeding indicates that the immediate fate of ingested protein is determined in part by the animal's previous carbohydrate intake. This observation is in harmony with the view that nitrogen metabolism in the animal is in a 'continuing' state, as recently pointed out by Borsook and Keighley ('35).

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FURTHER STUDIES CONCERNING THE FORMATION OF THE B-VITAMINS IN THE DIGESTIVE TRACT OF THE RAT[1]

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In previous publications (Guerrant and Dutcher, '34 a, b) it was shown that, under certain experimental conditions, dextrinized cornstarch exhibited unusual dietary properties when compared with sucrose or with the cornstarch from which the dextrinized starch was prepared. The unusual growth responses made by young rats while receiving diets deficient in the vitamin B complex and containing dextrinized cornstarch as the source of carbohydrate have been definitely associated with a characteristic condition existing in the digestive tract of these animals. It has been demonstrated that under such conditions the rat is able to synthesize, in the lower digestive tract, such dietary essentials as are necessary to supplement the vitamin B complex deficient diet. However, in order for the rat to obtain maximum benefits from the products thus synthesized, it is necessary that the animal ingest its excreta, especially the feces.

We have shown (Guerrant, Dutcher and Tomey, '35) that the elaboration of these dietary supplements, by the rat, takes place in the region of the cecum and it was suggested that this elaboration is probably due entirely to the action of certain microorganisms. The marked regularity of the synthesis of these dietary supplements when the rat receives the dex-

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trinized cornstarch diet, and the almost complete lack of such synthesis when comparable diets containing either sucrose or cornstarch are fed, led us to conclude that the above phenomena are due indirectly to differences in rapidity of digestion and subsequent absorption of these carbohydrates. Although it has been frequently observed that, under comparable conditions, greater quantities of dextrinized cornstarch reach the cecum than do either sucrose or unheated starch, much remains to be learned concerning the physiological processes associated with the digestion and the utilization of various forms of carbohydrates. The brief experiments herein reported were carried out with the view of obtaining further information concerning this interesting and important problem.

EXPERIMENTAL

The investigations of Whipple and Church ('35) have suggested that the marked contrast in the deportment of young rats receiving vitamin B complex deficient diets containing starch, as compared to other rats receiving comparable diets containing sucrose as the source of carbohydrate, might be due to the small quantity of fatty materials held by the starch particles. Such an explanation might appear to be adequate insofar as accounting for the differences in the nutritive properties of dextrinized starch and sucrose, but is wholly inadequate to account for a similar difference in the nutritive behavior of the dextrinized starch diets and the untreated starch diets, the latter being more comparable to sucrose-containing diets in their nutritive behavior than to the dextrinized starch diets. It does appear, however, that an appreciable quantity of fat in the diet might affect the carbohydrate digestion, thus changing the nature of the micro-flora of the lower intestinal tract, thereby affecting the synthesis of the dietary supplements. In fact it would seem that the inclusion of any substance or substances in the diet which would retard the absorption of the carbohydrate from the digestive tract might be a contributing factor.

In order to study the influence of definite quantities of fat on the synthesis of the dietary substances in the digestive tract of the rat, three diets containing sucrose as the source of carbohydrate were used (diets 369, 370 and 371). As will be observed from table 1, these diets were of similar composition and differed only in that diets 370 and 371 contained 10 and 20%, respectively, of hydrogenated cottonseed oil in place of an equal weight of sucrose.

TABLE 1

Giving the percentage composition of the various diets used in the investigation

DIETS	349	356	369	370	371	372	373	374	375	376
Purified casein	18	18	18	18	18	10	10	18	18	18
Salt mixture	4	4	4	4	4	4	4	4	4	4
CellU flour	2	2	2	2	2	2	2	2	2	2
Cod liver oil	2	2	2	2	2	2	2	2	2	2
Dextrin (autoclaved 4 hours)	74	0	0	0	0	0	0	0	0	0
Cornstarch	0	74	0	0	0	0	0	0	0	0
Sucrose	0	0	74	64	54	0	0	0	0	0
Hydrogenated cottonseed oil	0	0	0	10	20	0	0	0	0	0
White bread	0	0	0	0	0	82	0	0	0	0
Brown bread	0	0	0	0	0	0	82	0	0	0
Corn flakes	0	0	0	0	0	0	0	74	0	0
Dextrin (autoclaved 8 hours)	0	0	0	0	0	0	0	0	74	0
Dextrin (autoclaved 12 hours)	0	0	0	0	0	0	0	0	0	74
	100	100	100	100	100	100	100	100	100	100

Since the inclusion of dextrinized starch (starch which had been moistened with 0.1% solution of citric acid, autoclaved for 4 hours under 15 pounds of pressure and dried) in the diet of the rat had been found to result in a very marked increase in the biological value of the feces, it appeared important to know whether further autoclaving of the starch would bring about more marked changes in this direction. To test this out, four equal portions of cornstarch were weighed out, of which three portions were mixed with equal quantities of the dilute citric acid solution in the usual manner and autoclaved under 15 pounds of pressure for 4, 8 and 12 hours, respectively. The three portions of autoclaved starch

and a fourth portion of untreated starch were placed simultaneously in a large drying oven where they were kept until rendered free from moisture. When dry, each of the four portions was pulverized and incorporated into comparable diets (diets 356, 349, 375 and 376).

The better nutritional deportment observed among those animals which received the dextrinized starch diets in earlier experiments was apparently due to certain changes in the carbohydrate brought about by its treatment with heat. It, therefore, seemed possible that similar changes in the carbohydrate fraction might be brought about in the baking of bread. If such happened to be the case, a well-browned loaf might be expected to exhibit superior nutritional value. In order to investigate this particular problem, forty fresh loaves of bread, purchased on the local market, were used. Each loaf of bread was separated into two portions; the brown crusty exterior and the white spongy interior. The two bread portions were dried at room temperature, pulverized and incorporated into diets 372 and 373 as sources of carbohydrate. In order to have the protein content of these diets comparable to those previously mentioned, the casein content was reduced from 18 to 10%, as will be observed from table 1.

Continuing the reasoning along the same general line, it would appear that a highly dextrinized product such as corn flakes might exhibit very unusual dietary properties when fed to rats in vitamin B complex deficient diets as a source of carbohydrate. A quantity of this product was, therefore, purchased on the local market, dried, pulverized and incorporated in diet 374 in the proportions given in table 1.

In the nutritional studies each of the above described diets was fed to two comparable groups of young rats (six rats per group), in one group of which the animals had access to their own feces and in the other group the animals were denied access to any excreta. All animals were caged in individual metal cages which were provided with coarse screen bottoms. Fresh water and a liberal quantity of the respective

diet were kept before each animal at all times. Body weight changes and food consumption were recorded at weekly intervals. The feces from one group of animals receiving each diet were scraped from the cage pans, placed in a small earthenware container and returned to the cage daily. The feces from the second group of animals were removed from the cage pan daily, the number of pellets counted and then stored in group containers under ether, a different container being used for each group of rats every week. This procedure was followed for 6 weeks for all animals except those which died before the end of this period.

At the end of the experiment, all weekly collections of feces were extracted by shaking successively with four 100 ml. portions of ethyl ether, dried, weighed and their reducing value determined by the A.O.A.C. method ('24) for the determination of starch and other hydrolyzable carbohydrates of feeding stuffs.

Data

Although the experiments were continued for a period of 6 weeks, the mortality among certain groups of animals during the fifth and sixth weeks makes it difficult to compare the responses made by the several groups of animals during the entire experimental period. In order, therefore, to eliminate the factor of mortality from a consideration of the results, only the data obtained during the first 4 weeks of the experimental period are given. These data have been condensed and are given in table 2. The incidence of mortality during the last 2 weeks of the experimental period will be discussed in a subsequent paragraph.

DISCUSSION

It will be observed from the data presented in table 2 that the substitution of 10, or even 20% of hydrogenated cottonseed oil (diets 370 and 371) in the sucrose-containing vitamin B complex deficient diets, for an equal weight of the carbohydrate, resulted in no marked differences in the general

deportment of the experimental animals, except a measurable decrease in the amount of diet consumed. This decrease in food intake was to be expected because of the increased caloric value of the fat-containing diets. It will also be observed

TABLE 2

Showing the average deportment of each of the six animals composing the respective groups during the first 28 days of the experimental period

DIET NO.	GROUP NO.	DIETARY SUPPLEMENT	AVERAGE INITIAL WEIGHT	TOTAL CHANGE IN BODY WEIGHT	TOTAL FOOD INTAKE	DRY ETHER-EXTRACTED FECES				
						Total weight	Per cent	Number of fecal pellets	Average weight of fecal pellets	Reducing value of fecal matter
			gm.	*gm.*	*gm.*	*gm.*			*gm.*	*mg.*[1]
369	1	Feces	45	— 3	97	...	...	...	....	...
	2	None	46	—10	84	3.3	3.9	92	0.036	57
370	3	Feces	44	— 2	91	...	...	...	....	...
	4	None	45	—12	78	4.4	5.6	121	0.036	80
371	5	Feces	45	— 4	84	...	...	...	....	...
	6	None	44	—10	69	3.5	5.1	104	0.034	55
356	7	Feces	46	0	82	...	...	...	....	...
	8	None	44	— 7	76	3.3	4.3	103	0.032	43
349	9	Feces	46	22	108	...	...	...	....	...
	10	None	45	— 4	84	7.0	8.3	295	0.024	289
375	11	Feces	46	18	108	...	...	...	....	...
	12	None	46	— 1	90	7.9	8.8	332	0.024	292
376	13	Feces	44	20	107	...	...	...	....	...
	14	None	45	— 3	78	8.4	10.8	353	0.024	327
372	15	Feces	45	94	229	...	...	...	....	...
	16	None	44	90	196	9.4	4.8	287	0.033	230
373	17	Feces	45	73	179	...	...	...	....	...
	18	None	44	78	187	10.0	5.3	269	0.037	280
374	19	Feces	46	11	98	...	...	...	....	...
	20	None	46	0	86	5.8	6.7	220	0.026	138

[1] Reducing values are expressed in milligram equivalents of glucose.

from these data that those animals which had access to their feces (groups 1, 3 and 5) consumed more of the respective diets and lost less in body weight than did the corresponding groups of animals which did not have access to their feces (groups 2, 4 and 6). These relative differences were not

altered by the presence of hydrogenated cottonseed oil in the diet. It does appear, however, that the amount of ether-insoluble fecal matter was increased somewhat as a result of substituting this particular form of fat for an equal weight of sucrose.

It appears, therefore, from these data that young rats receiving vitamin B complex deficient diets which contain sucrose as the source of carbohydrate are benefited somewhat by having access to their own feces during the first week or 10 days of the experimental period. After this time, such feces appeared to have no supplementing value since the test animals declined in weight in a manner similar to that of the rats which did not have access to the excreta. The presence of 10 or even 20% of hydrogenated cottonseed oil in such diets did not alter the results with respect to rate of growth or maintenance of life. Mortalities among the animals receiving the sucrose diets were restricted to groups 2, 4, 5 and 6 and were five, six, two and four animals, respectively, all of which died during the fifth and sixth weeks of the experimental period.

When commercial cornstarch is moistened with a dilute solution of citric acid, autoclaved for 4 hours under 15 pounds of pressure and subsequently dried, some marked changes are brought about in the dietary properties of this substance. This fact may be brought out by comparing the results obtained from animals receiving diets 356 and 349. The general responses of those animals which received the diet containing the untreated starch (diet 356) were comparable to the responses made by animals receiving the sucrose diets, while those animals which received the dextrinized starch diet (diet 349) exhibited a much more favorable nutritional picture. As has been previously reported, those animals which received the dextrinized starch diet and which also had access to their own feces grew at a very favorable rate (22 gm. in 4 weeks), while those animals which received a similar diet, but did not have access to the feces, not only failed to grow but actually lost weight. This loss in weight was not so great, however,

as had resulted among comparable groups of animals which had received the starch or the sucrose diets.

Other noticeable changes, in addition to the increase in growth rate, resulted from the dextrinization of the starch. The percentage of ether-insoluble fecal matter was almost doubled, the number of fecal particles was trebled, the average weight of the fecal particles was reduced approximately one-fourth and the reducing equivalent of the fecal matter was increased sevenfold.

These data indicate quite clearly that dextrinization of cornstarch according to the method described, renders it less readily digested by the normal digestive processes of the rat. Such findings could not have been predicted from our present conception of carbohydrate hydrolysis and metabolism. The complete explanation for the lowered digestibility of the dextrinized cornstarch will have to await further studies. It appears, however, that during the process of dextrinization and subsequent drying, a portion of the carbohydrate fraction is, in reality, dehydrated or caramelized. When ingested by the rat, the caramelized carbohydrate must first become hydrated before it can be acted upon by the hydrolytic agents. This delay in digestion, along with the mild laxative effect of the caramelized products, tends to increase the amount of nutrients reaching the lower bowel and, in consequence, greatly encourages the growth of yeast or similar microorganisms. This appears to be the most probable explanation for the syntheses of the B-vitamins in the digestive tract of rats receiving dextrinized cornstarch diets.

Further autoclaving of the starch for 8 and 12 hours, respectively, did not greatly alter the nutritional properties of the starch beyond that which had been obtained by the 4-hour autoclaving (diets 375 and 376). There did appear to be some increase, however, in the per cent of fecal matter, in the number of fecal pellets and in the amounts of reducing substances in the feces, as a result of the longer periods of autoclaving. These increases were not great. The mortalities among the animals receiving the starch diet or the dextrin-

ized starch diets were restricted to groups 8, 10, 12 and 14 and were three, two, one and one rats, respectively, all of which died during the sixth week of the experiment.

The growth responses obtained as a result of feeding groups of young rats diets containing different parts of the baker's loaf (diets 372 and 373) were not according to expectation. It is highly probable, however, that these results were greatly influenced by the presence of considerable amounts of the B-vitamins in the original bread. All animals receiving the diets containing the bread made very unusual growth responses. In fact, the growth rate was so rapid that any beneficial effect of feces to those animals was obscured. The somewhat suppressed growth rate of those animals (groups 17 and 18) which received the diet containing the outer portion of the loaf (diet 373) indicates that the growth-stimulating value of the loaf had been reduced by the baking process. In all probability this reduced growth rate was due to a partial destruction of the B-vitamins near the exterior of the loaf.

The dietary supplementary value of the feces from those animals which received the diet containing corn flakes (diet 374) was quite definite, but not so marked as had been observed among those animals which had received the diets containing the dextrinized cornstarch diets. From the data obtained from the animals receiving this diet, as well as from those animals receiving the dextrinized starch diets (as contrasting those animals which had received the sucrose and the starch diets), it appears that a relatively large percentage of fecal matter of low density and of high reducing value is associated with the formation of substances in the digestive tract of the rat, which are effective in supplementing a vitamin B complex deficient diet. A large fecal output, composed of numerous pellets of relatively high reducing value, is believed to be indicative of a definite laxative effect in the digestive tract, while few fecal particles of low reducing value might indicate the opposite condition. In the above experiments, however, the average weight of the individual fecal particle

bore little if any relationship to their average size. The fecal pellets from the dextrin-fed rats were invariably large, fluffy and highly aerated, while those from the sucrose-fed rats were somewhat smaller in size and were very compact. The superior physical condition of the digestive tract of the dextrin-fed rats, as contrasting the sucrose-fed rats, was always apparent.

The recent contribution of Bliss and Green ('36) has greatly extended our knowledge concerning the phenomenon of 'refection.' While it is generally considered that refection and those conditions which cause coprophagy to vitiate experimental results are different phenomena, there remains the possibility of some relationship existing between the two conditions. In fact, several factors point toward the existence of such a relationship. While it is entirely possible that the two phenomena are caused by different types of microorganisms, it is also possible that both are caused by the same type of organism but in different parts of the rat's digestive tract. It seems reasonable to suppose that in the case of refection, where there is a direct absorption of the growth-stimulating factors, the elaboration must take place in the upper digestive tract, probably in the small intestines. Since in the other case, it is necessary that the rat ingest the excreta in order to receive full benefit of the products formed, this suggests that the elaboration takes place in the lower gut. The fact remains that in one case the supplementary substances are absorbed directly from the digestive tract, while in the other it is necessary that the animal re-ingest the feces in order to receive full benefit from the products synthesized in the rat.

SUMMARY

Further studies have been made concerning the role of the diet in the formation of the B-vitamins in the digestive tract of the rat. From results obtained during this investigation, the following conclusions are drawn:

1. The presence of 10 or 20% of hydrogenated cottonseed oil in the vitamin B complex deficient diet, containing sucrose as a source of carbohydrate, does not lead to a more favorable production of the B-vitamins in the digestive tract of the rat.

2. Further autoclaving of the starch beyond the usual 4-hour period does not lead to a significant increase in the amount of supplementary substances eliminated by the rat in its feces.

3. Those diets which, when fed to rats, result in relatively large percentages of fecal matter, having a low density and a high reducing equivalent, are conducive to the formation of substances in the digestive tract which are effective in supplementing a vitamin B complex deficient diet.

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THE INFLUENCE OF LACK OF VITAMIN A IN THE DIET ON THE PHAGOCYTOSIS PROMOTING PROPERTIES OF THE BLOOD SERUM[1]

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ONE FIGURE

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In spite of the fact that one of the outstanding symptoms occurring in vitamin A deficiency is xerophthalmia and that not infrequently other infectious processes are observed during the disease, the effect of vitamin A on those immunological processes which play a major role in the defense of the organism against infections are far from being clear. There are only very few investigations dealing directly with the immunological properties of the blood serum under conditions of vitamin A deficiency. Zilva ('19) studied amboceptor and agglutinin reactions in vitamin A deficiency and found them unaltered. Findlay and MacKenzie ('22) investigated the phagocytic index in vitamin A deficiency and found no deviations from the control. Werkman ('23) reported on a phagocytosis study in vitamin A deficiency which was also negative. All these investigations were performed on an insufficient number of animals. Moreover, the significance of the variations in the phagocytic index which did occur is not clear since the variations occurring in controls were not studied. It seems doubtful whether any conclusions can be derived from this experimental material. A reinvestigation of the immunological properties of the blood serum under the conditions of vitamin deficiency and particularly vitamin A defi-

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ciency seems to be very important since "a considerable reduction in resistance to infection occurs in animals suffering from a marked and prolonged deficiency of the fat soluble vitamins" (Robertson, '34). It should concern mainly the process of phagocytosis of which Arkwright and collaborators ('31) say that "the all important fact in relation to immunity is the development of thermostabile specific antibodies which promote phagocytosis." Before we accept the idea that the immunological properties of the serum are unaltered under the conditions of vitamin A deficiency and that the infections occurring under these conditions are secondary to the alterations of the epithelium of the skin and mucous membranes (Mori, '22; Wolback and Howe, '25) an extensive study of phagocytosis with modern methods seems imperative.

Such an investigation was conducted during the past 2 years in which, after several hundred control experiments, the phagocytosis method of Hamburger ('27) yielded results of a very high degree of accuracy.

METHOD

The experiments were carried out on fifty-seven vitamin A deficient rats and twenty-two control rats during March and April, 1935, and January and April, 1936.

Technic of phagocytosis study. The leukocytes were obtained from rats which had been injected with 25 cc. of a saponin solution 1:50,000 intraperitoneally. Sixteen hours later a second intraperitoneal injection of 25 cc. of 0.9% NaCl was made and 2 hours later the rat was killed and the abdominal cavity was washed with 100 cc. of citrate-sodium chloride solution. The fluid was filtered through a double layer of gauze and the number of leukocytes present in 1 cubic millimeter was ascertained. This method yielded a total of 100,000,000 to 150,000,000 leukocytes. The leukocytes were centrifuged off and the citrate-sodium chloride solution was decanted and washed twice with 0.9% sodium chloride. The experimental animals and the controls were killed and the blood was obtained from heart or carotid artery and collected in a centrifuge tube. It was allowed to coagulate

and the serum was used for the experiment. Test tubes fitted with ground glass stoppers were filled with the following solutions.

0.6 cc. starch suspension in 0.9% sodium chloride[2]
0.1 cc. m/15 phosphate buffer pH 6.8
0.1 cc. 0.9% sodium chloride
0.1 cc. serum diluted 1: 5 with 0.9% sodium chloride
0.1 cc. leukocyte suspension in sodium chloride

The mixture was shaken and the test tubes closed with the well-greased glass stoppers. They were placed immediately in rotating cylinders in an incubator which was kept at 37°. An apparatus similar to that described by Robertson and collaborators ('24) was used. The tubes were kept rotating in the incubator for from 25 to 45 minutes. Thereafter they were removed, shaken, placed on a rack, and 1 drop of a 10% formalin and 2 drops Lugol solution were added to the solution. Then the percentage of leukocytes which had ingested starch particles was determined in samples from the control and the experimental tubes. More than 200 cells were counted in every tube, but not infrequently twice as many cells were investigated. The accuracy of this improved Hamburger method is evident from the table 1, which shows the phagocytosis promoting power of two control sera in a number of experiments. It indicates that the variations in the phagocytosis promoting properties of two sera obtained from normal rats is generally less than 10% and very frequently even less than 5%.

The rats used for this experiment weighed about 40 gm. each. The diet consisted of:

	%
Casein	18
Salt mixture	4
Fleischmann's irradiated dried yeast	8[3]
Starch	65
Corn oil	5
Total	100

[2] The starch particles were of a uniform size, approximately half as large as the leukocytes. We had about one particle for each leukocyte in each preparation.

[3] The irradiated yeast contained 10 D = 400 international units per gram. The daily consumption of vitamin D was approximately 15 D or 600 international units.

The salt mixture consisted of:

NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4(H_2O)$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Ferric citrate $\frac{1}{2}$ H_2O	0.118
Calcium lactate	1.3
	3.698

TABLE 1 [1]

Control experiments. Comparison of the phagocytic index of sera of two normal rats. Phagocytic index

EXPERIMENT NO.	RAT I	RAT II	DIFFERENCE IN PER CENT
1	49.2	49.6	+1
2	63.9	62.2	—2.3
3	37.0	39.3	+6.3
4	29.0	30.7	+6.0
5	45.2	46.7	+3.5
6	60.8	65.1	+7.0
7	65.5	68.9	+5.2
8	63.4	66.5	+5.0
9	65.0	67.7	+4.2
10	66.6	64.1	—3.6
11	38.4	35.6	—7.0
12	47.8	46.6	—2.3

[1] Phagocytes obtained from a rat by the procedure described above were incubated in two different sera (rats I and II) and the percentage of leucocytes which had ingested a starch particle was determined (phagocytic index). Column 4 shows the difference in per cent if the phagocytic index of the serum of rat I is taken as 100.

The differences in the absolute values of the phagocytic indices of the ten experiments of table 1 are due to the fact that the incubation time varied in different experiments between 20 and 40 minutes.

In the case of the controls Smaco, as a source of vitamin A, was added (2.5 cc. to 3333 gm. of food). It contains 7500 U.S.P. units per gram.

RESULTS

Table 2 indicates that the animals were sacrificed for the purpose of the phagocytosis experiment after growth had ceased completely and a distinct loss in weight was established. All experimental animals showed typical signs of

TABLE 2

The phagocytosis experiments, 1935

DATE	INITIAL WEIGHT	MAXIMUM WEIGHT	FINAL WEIGHT	WEIGHT LOSS IN PER CENT	DURATION OF EXPERIMENT IN DAYS	PHAGOCYTOSIS, PER CENT			PHAGOCYTOSIS IN PER CENT OF CONTROL
				A. Control rats					
4/11	33.5	135.8	135.8	0	40	356[1]/560[2]	63.5	66.2	100
	32.0	151.9	151.9	0		335 /482	69.5		
				B. Vitamin A free rats					
4/11	37.6	124.0	96.2	22.4	40	65/200	32.5		39.2
	41.5	134.2	104.2	22.0		125/175	71.2		107.8
	38.3	128.3	92.1	28.2		200/305	65.5		99.0
	36.9	133.0	113.4	14.5		120/200	60.0		90.8
	31.3	100.8	79.0	21.5		120/240	50.0		75.6
	35.1	92.5	84.7	8.3		110/235	46.7		70.7
	39.1	140.8	114.5	18.4		93/200	46.5		70.3
	39.0	140.3	122.5	12.5		120/205	58.4		88.4
				A. Control rats					
4/13	38.5	148.0	148.0	0	42	130/200	65.0	65.2	100
	34.8	139.1	139.1	0		135/207	65.4		
				B. Vitamin A free rats					
4/13	38.5	123.2	104.4	15	42	250/415	60.4		92.6
	38.9	122.5	94.7	22.7		123/194	63.2		92.5
	33.0	116.3	104.8	9.7		220/367	60.1		92.4
	36.8	116.5	102.3	11.7		250/325	77.1		118.5
				A. Control rats					
4/17	31.5	173	173	0	46	325/570	56.9[1]	56.9	
	40.5	174	174	0		132/232	56.8		
				B. Vitamin A free rats					
4/17	37.7	139	112	19.2	46	400/595	67.4		118.5
	32.4	110.3	84.6	23.3		185/245	75.6		133.0
	33.3	119.6	84.8	29.0		259/464	56.0		98.5
	39.2	126.5	88.6	30.0		185/295	62.8		110.8
				A. Control rats					
4/22	39.0	198	198	0	51	320/567	56.5		
				B. Vitamin A free rats					
4/22	41.8	122.3	81.3	23.6		5/110	4.6		8.2
	34.0	104.5	68.4	35.0		285/385	74.1		131.8
	37.6	106.0	82.8	21.8		200/350	57.3		101.5
	38.0	129.5	82.2	36.5		142/247	57.6		102
	36.6	125.7	100.3	19.7		251/335	75.1		133.0
				A. Control rats					
4/27	32.0	165.8	164.5	0.5	55	58/148	39.2	37.5	
	37.0	210.0	210.0	0		128/348	36.9		
				B. Vitamin A free rats					
4/27		117.5	91	22.5		55/285	19.3		51.6
		111.4	86.8	22.1		54/164	32.9		87.9
		128.4	90	30		56/256	21.9		58.5
		122.2	86.8	29		37/157	23.6		63.1
		132.2	94.2	29		45/155	29.0		77.5
		112.5	80.5	28.3		43/153	28.2		75.3
		107.8	77.5	28		41/161	25.6		68.4
		118.0	81.8	30.6		30/230	13.1		34.8

[1] Number of leucocytes containing starch particles. [2] Total number of leucocytes counted.

vitamin A deficiency. Particularly marked was xerophthalmia, alterations in the skin around the toes, a cyst at the back of the tongue, inflammatory processes of the genital organs. Table 2 presents the results of the first phagocytosis experiment which comprises twenty-nine experimental animals and eleven control animals. This experiment was carried out during 5 days after the experimental animals had been on the vitamin A free diet for from 40 to 55 days. The changes in phagocytosis are expressed in percentages of the controls. The variations in the phagocytosis of the controls are extremely small.

In this group of experiments those animals which showed the most marked loss in weight were sacrificed first. The experiments showed that the alterations in phagocytosis were not uniform. In the first experiment, April 11, 1935, four or five of the eight experimental animals showed a very marked drop in the phagocytosis promoting power of the serum. In the following three experiments we observed either no change or an increase in the phagocytic index, but in the last experiments phagocytosis was markedly decreased. As will be seen later, the second experiment carried out in the following year gave similar results. Obviously the phagocytic index is the result of at least two opposing factors. It seems to be very likely that under the influence of the infections which actually take place in vitamin A deficient rats the phagocytosis promoting properties of the serum are increased just as they are in any other infections under ordinary conditions, but if the conditions of vitamin A deficiency continue the ability of the animal to produce these immune bodies is greatly disturbed. In such cases we find a considerable decrease in phagocytic index in spite of the fact that these animals have fully developed signs of vitamin A deficiency and severe inflammatory processes in various parts of the body.

In regard to details, it may be seen quite clearly from table 2 that there is no relationship between the weight loss and the phagocytic index, neither does a parallelism exist between the severity of the clinical symptoms and the phagocytic index.

In view of the fact that it is to be expected that the resistance of different animals and the development of the active immune bodies in these animals show considerable temporal individual variations, it is easily understandable that the time at which

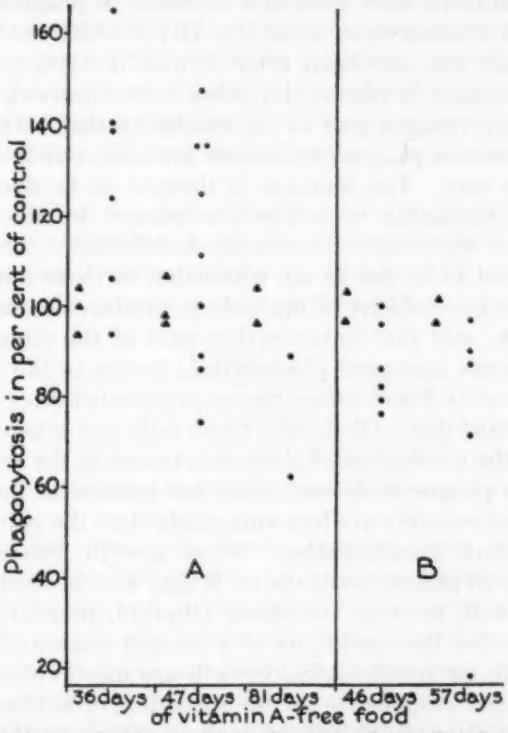

Fig. 1 The influence of a vitamin A free diet on the phagocytic index. Controls are represented by triangles and the indices of the experimental animals (vitamin A free diet) are represented by dots. The data shown in A and B were obtained in two different experiments.

a decrease in phagocytosis occurs varies for different experimental animals of the same group. There seems to be, however, as a rule, the tendency that the decrease in phagocytosis is more marked with increased duration of the vitamin A free period. This is clearly brought out in the second vitamin

experiment (fig. 1A) in which after 36 days of vitamin A free diet the phagocytic index was either increased or normal, after 47 days five animals showed an increased phagocytic index and three a decrease, and after 81 days the two experimental animals both showed a decrease in phagocytosis. In the third vitamin experiment (fig. 1B) in which the first group of animals was sacrificed after 46 and 57 days, respectively, only decreases in phagocytic index were observed.

The experiments lead to the conclusion that both increased and decreased phagocytic indices are observed in vitamin A deficient rats. The increase is thought to be due to an increased formation of antibodies induced by the infectious processes which occur in vitamin A deficiency. The decrease is believed to be due to an exhaustion of these antibodies or rather to the inability of the body to produce new ones. Thus it may be said that in the earlier part of the vitamin A lack experiments increased phagocytosis occurs in the face of infections as is found under the same circumstances in animals on a normal diet. Obviously, those cells and organs responsible for the production of those substances in the serum which promote phagocytosis and which are present in the serum in increased amounts are less vulnerable than the epithelial cells which show keratinization. Since growth ceases before a decrease in phagocytosis occurs it may also be said that those organs with internal secretions (thyroid, pituitary, thymus) which under the conditions of a normal vitamin diet are responsible for physiological growth are more vulnerable than the systems responsible for the formation of antibodies. This differential susceptibility of various organs in the organism to vitamin A deficiency explains the dispute of various authors on the question whether vitamin A is an anti-infective vitamin or not. The direct experiments which have been carried out to decide this question and which were mentioned in the introduction were mostly negative, but the results are, for reasons given above, unconvincing. The study of the resistance of animals to natural and artificial infections shows quite variable results. This, too, is understandable if one

takes into account the fact that, depending on the general state of health of the animals, the duration of the period of vitamin A deficiency and the mode of infection, the animals may exhaust their defensive forces at different times and to a different degree. We come, therefore, to the conclusion that in vitamin A deficiency of a degree leading to pronounced symptoms (cessation of growth, loss of weight, xerophthalmia, infectious processes on skin and mucous membranes) a normal or even an increased phagocytic index may occur. Upon further progression of the disease, however, a decrease is observed.

The fact that in the majority of cases (table 3) a decreased resistance to natural or artificial infections occurs in vitamin A deficient rats is well explained on the basis of our observations that finally vitamin A deficiency leads to a very marked

TABLE 3

Summary of all vitamin deficiency experiments

Number of animals: 57

11 animals show no change in phagocytic index
16 animals show increased phagocytic index
30 animals show decreased phagocytic index

decrease in the phagocytic index. It seems to be of importance to emphasize that results applicable to various types of bacterial infections can be arrived at on the basis of experiments which do not take the specific properties of the bacteria into account, but which are based on experiments on phagocytosis of an inert material (starch). Obviously we deal here with a factor common to all processes of phagocytosis and the results are not obscured by the specific properties of certain kinds of bacteria.

This discussion makes it clear that we believe that the action of vitamin A on phagocytosis is not a direct one on the leukocytes or the particles ingested, but on those cells and organ systems in the body which, under the influence of hormones and vitamins, produce those phagocytosis promoting properties of the serum which determine the phagocytic index.

In other words, we would not expect the addition of vitamin A to the serum in vitro to increase the phagocytic index. However, the performance of such an experiment is difficult because of the insufficient water solubility of the vitamin A.

If our observations are correct that during the development of a vitamin A deficiency in rats the phagocytic index increases or decreases it is to be expected that control values should be obtained again when such vitamin deficient rats are put on an ordinary diet for a length of time sufficient to cure the animals completely. Such experiments have been carried out with six

TABLE 4

Reversibility of the effects of vitamin A deficiency on phagocytosis

RAT NO.	NUMBER OF DAYS ON VITAMIN A FREE DIET	NUMBER OF VITA-OF UNITS MIN A INJECTED	NORMAL DIET DAYS	WEIGHT AT THE END OF THE VITAMIN A FREE PERIOD	WEIGHT AT THE END OF THE SUBSEQUENT PERIOD WITH NORMAL DIET	PHAGOCYTOSIS			PHAGOCYTOSIS IN PER CENT OF CONTROLS
VI, 11	61	1200	51	134	219	150/350	42.9		102.9
VI, 2	66	800	46	152	263	145/355	40.9		98.1
VI, 5	66	800	46	137	196	120/310	38.4		92.2
VI, 7	66	800	46	146	216	135/360	37.6		90.1
V, 5	83	400	29	131	171	245/620	39.6		94.7
V, 9	83	400	29	143	175	278/728	38.2		91.3
Control 1					250	269/628	42.8	41.7	100
Control 2					220	290/710	40.8		

rats, each of which showed marked symptoms of vitamin A deficiency at the end of 61 to 83 days of vitamin A free diet. The animals were treated by intraperitoneal injections of vitamin A and our standard diet as described above was given for 51 to 29 days (table 4). They were then apparently normal and were sacrificed and the phagocytosis promoting properties of their sera were determined. The phagocytic indices are nearly all within the limits of the controls, and the two which are below that value show insignificant differences. It may be said, therefore, that the phagocytosis promoting properties of the serum of rats subjected to vitamin A deficiency are altered

in a reversible fashion since a normal diet may restore control values.

We are cognizant of the fact that the infection itself may have been the sole cause of the exhaustion of the phagocytosis promoting properties of the serum. However, inasmuch as the return to normal diet promptly restores the health and the phagocytosis promoting properties of the serum of the animals we feel that vitamin A plays the major role.

Finally the question arises whether weight loss itself may not alter the phagocytosis promoting properties of the serum. This important question will be discussed in a subsequent paper. Suffice it to say here that much greater losses in weight than were observed in our vitamin A deficient animals are necessary in order to cause a decrease in the phagocytic index.

SUMMARY AND CONCLUSIONS

The following results were obtained in experiments carried out with an improved Hamburger method on the phagocytosis promoting properties of the serum under conditions of vitamin A deficiency.

1. Rats which under the influence of vitamin A deficiency developed marked clinical symptoms, such as loss in growth and body weight, xerophthalmia, infections of skin and mucous membranes, may show either increases or decreases in the normal phagocytic index. This is due to the fact that the infectious processes which occur under vitamin A deficiency elicit the production of antibodies to an increased degree during the earlier states of the vitamin A deficiency. Later on the production of new antibodies does not keep pace with the demands of the organism and, as a result, the phagocytic index decreases considerably. Since a fully developed vitamin A deficiency may be accompanied by an increased phagocytic index this vitamin does not seem to be indispensable for the production of antibodies. Upon further progress of the disease, however, the phagocytic index decreases and explains well the fact that such animals are more susceptible to infections than animals under normal diet.

2. Weight loss and severity of symptoms do not run parallel to the changes in phagocytic index.

3. The changes in phagocytic index are completely reversible when the vitamin A deficient animals are put on a normal diet for a sufficiently long period of time.

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PHOTOMETRIC DETERMINATIONS OF UREA, URIC ACID, CREATININE AND HEMOGLOBIN IN THE BLOOD OF SCORBUTIC GUINEA PIGS

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ONE FIGURE

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Two years ago, in connection with a study of the anti-scorbutic properties of galacturonic acid, concurrent analysis of the blood of scorbutic guinea pigs indicated that a more detailed investigation of this phase of the problem might produce data which would be of considerable diagnostic value (Johnstin and Potter, '35). In spite of the vast amount of work which has been done upon scurvy, the mechanism of the pathological progression of this disease is still uncertain. The study is complicated because the symptoms vary with different animals of the same species, and the rapid course of this disease in laboratory animals is quite different from the slow development in man.

The nitrogen metabolism of scorbutic guinea pigs has been studied by several investigators, including Palladin and Kudrjawzewa ('24), Jarussowa ('26, '28), Nagayama and Sato ('28), Shipp and Zilva ('28) and Tomita ('32), but the conclusions, based chiefly on the results of urine analysis, are not in agreement. It is the opinion of most workers in the field that scurvy is accompanied by deranged nitrogen metabolism and that there is pathological retention of nitrogenous wastes. From a study of the uric acid content of scorbutic urine, Nagayama (Nagayama and Sato, '28) con-

cluded that there was retention of this compound, and Tomita ('32) in a later paper published similar results. Shipp and Zilva ('28), however, after a detailed investigation of the nitrogen intake and output concluded that, in the absence of vitamin C, there was no evidence of deranged nitrogen metabolism, and that any changes in nitrogen levels were due to the accompanying inanition.

It is obvious that the question of the effect of scurvy on nitrogen metabolism cannot be settled until there are concurrent studies of the total nitrogen intake and the blood and urine nitrogen of scorbutic animals.

The literature contains very few references to analyses of the blood of scorbutic animals and the reason for this is attributed to the difficulties encountered in the collection of a sufficient number of blood samples during the experimental period. With the macro methods of analysis in common use such comparatively large quantities of blood are required that small animals would soon die of anemia. The introduction of micro methods, which can now be carried out with 0.1 and 0.2 cc. of blood, has made it possible to follow the course of diseases without undue depletion of the blood volume. However, even when the micro methods are used with small quantities of blood the errors are so large, when colors are compared in an ordinary colorimeter, that it is impossible to interpret the results correctly.

In the present study, some of these sources of error have been minimized by using the Pulfrich photometer for colorimetric comparison. After the proper color filter for the particular determination has been decided upon and a concentration curve has been prepared, successive determinations can be made with this instrument with remarkable accuracy and speed.

It was decided to confine this work, at first, to the determination of those nitrogenous constituents of the blood which have previously been studied in the urine of scorbutic animals and reported by other workers, namely, uric acid, creatinine, urea, and also hemoglobin. The consensus of opinion, as

expressed by Myers, Fine and Lough ('16) is that the determination of the individual constituents of blood and urine gives much better insight into kidney function than does the measurement of the total non-protein nitrogen. This last determination when carried out photometrically with micro quantities, gives consistently high results, 30 to 38 mg. % of total non-protein nitrogen, in both normal and scorbutic blood. These results are not reported in detail at this time because the interpretation is uncertain. It should be explained, however, that in the procedure for the determination of non-protein nitrogen the sulfuric acid digestion and evaporation are followed by the use of Nessler's reagent. The resulting solution, although apparently clear in daylight, is distinctly turbid in the strong light of the photometer. It was thought that the high readings might be due to this slight turbidity, and not necessarily to high non-protein nitrogen content.

EXPERIMENTAL

Ten guinea pigs of fairly uniform size were selected and were adjusted to Eddy's ('29) modified Sherman, LaMer and Campbell basal ration. This diet was supplemented with green vegetables and tomato juice until it was determined that all of the animals were growing well. Six of the guinea pigs were then chosen for the experimental scurvy group and were fed the vitamin C free diet. Four others were fed the same diet and were given tomato juice, in addition, to provide the vitamin C. Unfortunately, two of the experimental animals were lost at an early date but the others followed the true scurvy course without complications. It was soon determined that the complete blood study could not be made during the short period of acute scurvy; consequently, the lives of the experimental animals were prolonged with doses of tomato juice at 4-day intervals. This lessened the precipitous growth decline and made possible a greater number of blood determinations. In this way the lethal stage was reached after a 6-week period rather than after 3 weeks. The growth curves of each animal showed a typical scurvy

picture and this was further substantiated by the gross lesions which were observed at autopsy. Figure 1, which follows, gives the individual growth curves of all the experimental animals. It is obvious that each of these follows the usual course.

The blood samples were taken from the ears of the guinea pigs, by capillary pipettes, after a small incision was made at the margin. The same ear was never used more than twice a week but several samples could be obtained from one in-

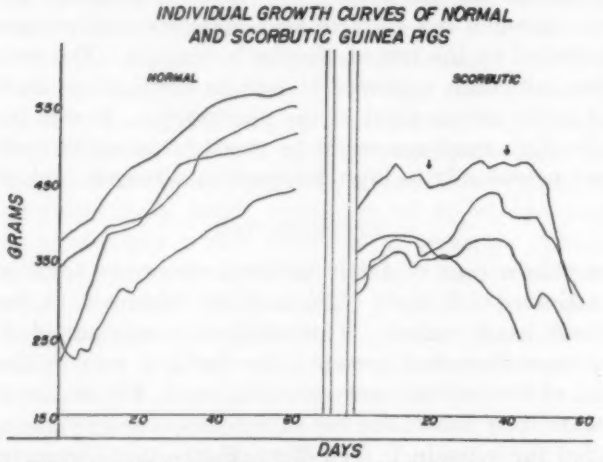

Fig. 1 The arrows on the scorbutic growth curves indicate the time when small doses of tomato juice were given to extend the scurvy period.

cision. Hemoglobin determinations were made at the same time, the blood being collected in a 5-cc. capillary diluting pipette. Throughout the work duplicate determinations were made frequently so that there could be no question as to the accuracy of the results. This was done both by the use of different portions of the same filtrate or, when necessary, with different blood samples taken at the same time. A total of 157 samples of blood was analyzed.

For each of the four constituents determined the procedure was as follows: a color absorption curve was first prepared

by determining the per cent of transmission with all of the nine color filters of the Pulfrich photometer. A filter showing suitable absorption was then selected, and a concentration curve was prepared by the use of standard solutions of three different concentrations. (In the case of hemoglobin a Newcomber plate was used instead of the standard solutions.)

TABLE 1

Blood determinations from animal no. 2—a normal subject

DATE	WEIGHT	HEMOGLOBIN	UREA NITROGEN	URIC ACID NITROGEN	CREATININE NITROGEN
	gm.	%	*mg.* %	*mg.* %	*mg.* %
3/ 5/36	302	93.0			
8	308				
11	326				
14	350				
17	360				
20	368		17.0	2.10	2.90
23	380				
26	391				
29	392				
4/ 1/36	401				
2	407				
4	...		15.0		3.0
5	...				
6	409				
7	...				
8	412				
9	...				
10	...	84.6	21.8		
11	416			2.50	
14	421		16.6		
16	426				
17	430				
4/18/36	439	83.9			

If the absorption of the colored solution follows the Lambert-Beers law, this curve should pass through the origin for zero concentration. With such a curve, the concentration of an unknown solution can be read directly, after the per cent of transmission is obtained in the photometer.

Urea. The standard calibration curve for urea nitrogen was made with different quantities of a standard ammonium

sulfate solution. The yellow color was produced according to Folin and Svedberg's ('30) method with Nessler's reagent. Color filter S 43 and the 10-mm. cup were used to make the transmission readings. The gum ghatti which is recommended

TABLE 3

Blood determinations from animal no. 6—scorbutic subject

DATE	WEIGHT	HEMOGLOBIN	UREA NITROGEN	URIC ACID NITROGEN	CREATININE NITROGEN
	gm.	%	*mg. %*	*mg. %*	*mg. %*
3/ 5/36	355				
8	365				
11	377	95.8			
14	401				
17	417	84.7			
20	437			2.30	
23	436			2.35	
26	409				
29	405				5.2
4/ 1/36	407	90.8	20.0	1.70	
2	...				
3	...		22.4	2.05	
4	407				
5	...			1.95	
6	394	89.7	18.0	1.65	
7	384		21.0		3.8
8	...				
9	...				
10	...				
11	356	88.0		1.40	3.6
12	...				
13	...				
14	334	66.0		1.95	
15	314			1.60	
16	306	71.9			3.0
17	300	75.9	19.0	2.30	
4/18/36	...			1.37	

in the Folin micro method was found not to be necessary for the comparative readings. If it is used, the colloidal appearance produced must be compensated by adding an equal quantity of the gum ghatti to the water solution which is placed in the comparison cup.

Uric acid. The calibration curve for uric acid nitrogen was obtained by making transmission measurements of different quantities of a standard uric acid solution with filter S 57 and a 10-mm. cup. The actual determination was performed as prescribed by Folin's ('33) newest micro method. Only 0.1 cc. of blood was used but the required depth of color was obtained by decreasing the dilution one-half.

Creatinine. The creatinine determination, at first, caused considerable trouble. The usual method of producing the red creatinine picrate by the use of alkaline picric acid required so much picric acid, that transmission readings could not be made in the photometer. The light was either entirely absorbed, or entirely transmitted. By reducing the concentration of the picric acid, it was found that filter S 50 would give transmission readings below 80% and above 5%. A corresponding dilution of the centrifuged filtrate was made by diluting 4 cc. of filtrate with 1 cc. of water. To this was added 3 cc. of an alkaline picrate solution (10 cc. 2% picric acid + 1 cc. 10% sodium hydroxide). The total volume was 8 cc. The correction for the absorption of the red isomer of creatinine picrate was made by using a standard solution of creatinine chloride treated with alkaline picric acid as described above and an alkaline picric acid blank in which water was used in place of the filtrate. Each had the total volume of 8 cc. The decreased transmission of the creatinine solution through color filter S 50, compared with the transmission of the picric acid blank was a measure of creatinine present. With larger concentrations of creatinine, longer standing was required to develop the full color, but the logarithmic curve was easily obtained.

Hemoglobin. The hemoglobin was determined with the aid of the standard Newcomber plate, but, instead of using this in the ordinary colorimeter, it was inserted in a frame and used as a standard for the photometric measurement. The calibration curve for the percentages of hemoglobin lay along a straight line between two limiting points on a logarithmic scale: the point for 100% hemoglobin, in which case the

standard and the sample gave identical readings at 100% transmission, and the point for 0% hemoglobin in which the transmission of the standard compared with water (0% hemoglobin) was only 51.7% for color filter S 50. With this modification, the photometric results proved much more accurate

TABLE 3

BLOOD DETERMINATIONS, DATES	HEMOGLOBIN		UREA NITROGEN		URIC ACID NITROGEN		CREATININE NITROGEN	
	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic	Normal	Scorbutic
	%	%	mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
3/ 8/36	93.0	89.0						
11	95.0	90.3						
14		88.7						
17		88.7	17.0			2.30		
20			15.0		2.10	2.21	2.90	
23					2.00	2.27	3.3	3.0
26					2.50			
29	94.0				1.63	1.87		
4/ 2/36		91.5		21.0		1.85		3.4
5	93.9	90.1	16.0	18.2		2.18	3.0	
8		87.0		16.8		1.78		2.9
11	84.6	87.3	18.9	16.6	2.30	1.58		3.3
14		78.1	16.6	19.0		2.06		3.2
17	89.0	73.9		17.8		2.07	2.85	2.9
Standard solution used in calibration	Newcomber glass standard		Ammonium sulfate, 1 cc. 0.01 mg. N		Folin's uric acid standard, 1 cc. 0.002 mg. uric acid		Creatinine chloride, 1 cc. 0.001 mg. creatinine	
Color filter used for the calibration	S 53		S 43		S 61		S.53	
Stratum thickness	10 mm.		10 mm.		10 mm.		10 mm.	

than identical measurements with the ordinary colorimeter when the same concentrations were used.

Tables 1 and 2 give a complete summary of the study made on a normal animal and on a scorbutic animal, respectively. These are typical of the results obtained for all the animals in each group.

Table 3 has been constructed to give a brief comparative picture of the two groups during the entire experimental

period. It gives average results of the determinations made on the animals of each group at the same stage in the experiment.

CONCLUSIONS

The work reported in this paper shows that the urea, uric acid, and creatinine in the blood of scorbutic guinea pigs did not rise above the normal level, and, therefore, no indication of abnormality in nitrogen metabolism was found in these animals. This substantiates the conclusions of Zilva (Shipp and Zilva, '28) and his co-workers, based on urinary studies, that there was no evidence of deranged nitrogen metabolism. On the other hand, these results are at variance with the theories of certain investigators whose work has been previously mentioned. Jarussowa ('26, '28), from her experiments with urine, thought that the nitrogenous waste products were not excreted properly during the scorbutic period. Insufficient excretion implied accumulation of these products in the blood, but such increases have not been found. No evidence has been found, either, to support Nagayama's (Nagayama and Sato, '28) suggestion that the decrease in uric acid in the urine of scorbutic guinea pigs resulted from the accumulation of that waste product in the blood.

In the case of creatinine, the values found for both groups of animals are consistently somewhat higher than those usually reported as normal. This point is of interest in connection with the suggestion of Behre and Benedict ('35) that the chemical reaction involved in the creatinine-picrate method is not dependable.[1]

The results on hemoglobin are not in agreement with those of other workers, notably Mettier and Chew ('32), and Mouriquand, Leulier and Michel ('25) who have reported very serious anemias in scorbutic animals. The hemoglobin decreased, as was expected, but the anemia produced was never severe. This was interesting since the life of the animals was purposely prolonged in order to allow the slower development of scurvy and to give ample time for the study

[1] The new method proposed by these authors (Benedict and Behre, '36) was published after the completion of the present study.

of such accompanying pathological symptoms as anemia. It is believed that the hemoglobin technic employed in this work made it possible to detect any variations in hemoglobin more accurately than was possible by any methods which we have used heretofore.

Grateful acknowledgment is made to the department of zoölogy and physiology of Wellesley College for their cooperation in this work.

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